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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CARBOHYDRATES

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I. Introduction ..... 17
II. Instrumentation and Stationary Phases ..... 18
1. Chromatographic Equipment ..... 18
2. Stationary Phases ..... 23
III. Separations and Applications ..... 32
1. Analytical Separations ..... 32
2. Additional, Selected Applications ..... 50
3. Preparative, Liquid Chromatography ..... 58
IV. Special Aspects and Problems ..... 63
1. Detectability and Accuracy ..... 63
2. Combined L.C. Techniques (L.C.–M.S., and L.C.–N.M.R. Spectroscopy) ..... 69
3. Separation of Carbohydrate Anomers ..... 70
4. Future Trends ..... 71
Addendum: References Since 1986 ..... 327

## I. Introduction

This chapter covers advances in so-called high-pressure or high-performance liquid chromatography (often abbreviated as h.p.l.c.) of carbohydrates. The term l.c. is used here to designate the rapid (<1 h) separation of carbohydrates in modern, liquid-chromatography systems, which include a high-performance, solvent-delivery system using one or more pumps, an injection valve, a column packed with 3–10-$\mu$m, monodisperse, resin- or silica gel-based particles, and a high-performance detection-system. These systems are operated at elevated pressures that, depending on the various stationary and mobile phases, the size of the columns employed, and the flow-rates used, may range from ~1 to >30 MPa. In this chapter, l.c. separations of almost all mono-, di-, and oligosaccharides are discussed from both a theoretical and a practical point of view. In addition, the general principles involved in the care and mainte-

nance of the relevant instrumentation and stationary phases are covered. A separate Section on preparative l.c. methods for carbohydrates has been included, and this is the first article to treat this important and growing subject. Other Sections, which provide solutions to problems of detectability and of peak broadening (resolution of anomeric forms) are included, and the current status of emerging l.c. techniques (l.c.–m.s., and high-performance affinity chromatography) are considered. High-performance, size-exclusion chromatography of oligo- and poly-saccharides will not be discussed here, but it has been described in two reviews.[1,2]

It is assumed that the reader is familiar with such common chromatographic concepts as efficiency, selectivity, capacity factors, and theoretical plates, and how these parameters affect and effect chromatographic resolution. Excellent descriptions of these general chromatographic principles have been published.[3,4] Other reviews on various aspects of carbohydrate separations will be cited in the appropriate Sections.

## II. Instrumentation* and Stationary Phases

### 1. Chromatographic Equipment

**a. Solvent-delivery Systems.**—Almost all modern, commercially available pumps and controllers are useful for this kind of carbohydrate analysis. Some, however, because of aspects specifically related to carbohydrate applications, are more useful than others. A majority of the l.c. methods described here required refractive-index detectors, and these instruments are sensitive to changes in solvent flow, pressure, and composition. Hence, the most useful pumping systems are those that deliver pulse-free and precise solvent-flow. Although most solvent-delivery systems are capable of blending two or more solvents, to afford isocratic mobile phases, few of these systems can blend these solvents accurately, and when the columns on these systems are monitored by refractive index detectors, extremely unstable baselines are the result. Hence, many chromatographers must tediously pre-mix their solvents in order to obtain

(1) S. C. Churms, in E. Heftman (Ed.), *J. Chromatogr. Library*, Elsevier, New York, 1983, pp. B223–B286.
(2) S. C. Churms, in G. Zweig and J. Sherma (Eds.), *Handbook of Chromatography, Carbohydrates*, Vol. I, CRC Press, Boca Raton, FL, 1982, pp. 69–129 and 175–187.
(3) K.-P. Hupe, in A. Henschen, K.-P. Hupe, F. Lottspeich, and W. Voelter (Eds.), *High Performance Liquid Chromatography in Biochemistry*, VCH Publishers, Deerfield Beach, FL, 1985, pp. 1–15.
(4) L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, 2nd edn., Wiley–Interscience, New York, 1979, pp. 15–82.

* Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

stable baselines. Some pumps are now available that are capable of accurately mixing solvents on line, and the purchase of these instruments is recommended.

Another consideration in choosing a solvent-delivery system is the matter of the maximum and minimum flow-rate ranges. For extremely precise solvent-metering in microbore-column applications, pumps capable of operating in the 50–100 $\mu$L/min range are useful. For preparative chromatography, "dedicated" preparative instruments[5,6] are available that can provide flow rates in excess of 100 mL/min. For most laboratory applications, however, it is not necessary to buy a dedicated microbore or preparative chromatograph, as l.c. systems are now available[7,8] that are capable of performing both functions with only minor changes in plumbing and hardware. Regardless of the type of solvent-delivery system used, solvents should, for best results, be degassed by vacuum, with helium, or by use of commercial degassing instruments. All of these degassing systems work well, but the last is the most convenient, and allows continuous operation.

**b. Equipment for Preserving Column-Life.**—Equipment in this category includes pre-columns, guard columns, silica saturator[9,10] columns, and various cartridges containing bonded-phase packings or ion-exchange resins. These can, and should, be used on- or off-line, to prevent sample contaminants from entering the analytical column. The appropriate use of saturator and pre-columns for each type of column stationary-phase will be given in the next Section. When pre-columns are installed instead of sample loops, they can be used as concentrators[11] for dilute samples, and can be readily backflushed, after injection, to remove unwanted sample-components.[12]

Bonded-phase silica and ion-exchange resins in plastic cartridges and mini-columns are very useful for off-line prepurification of samples, especially those for preparative chromatography, when appropriate pre- or guard columns may not be available for on-line clean-up of a sample.

(5) N. W. H. Cheetham and P. Sirimanne, *Carbohydr. Res.*, 96 (1981) 126–128.
(6) C. D. Warren, A. S. Schmit, and R. W. Jeanloz, *Carbohydr. Res.*, 116 (1983) 171–182.
(7) K. B. Hicks, S. M. Sondey, D. Hargrave, G. M. Sapers, and A. Bilyk, *LC Mag.*, 3 (1985) 981–984.
(8) K. B. Hicks and S. M. Sondey, *J. Chromatogr.*, 389 (1987) 183–194.
(9) D. L. Hendrix, R. E. Lee, Jr., J. G. Baust, and H. James, *J. Chromatogr.*, 210 (1981) 45–53.
(10) B. Porsch, *J. Chromatogr.*, 253 (1982) 49–54.
(11) J. A. Polta, D. C. Johnson, and K. E. Merkel, *J. Chromatogr.*, 324 (1985) 407–414.
(12) K. Mopper and L. Johnson, *J. Chromatogr.*, 256 (1983) 27–38.

These have been effectively used to remove lignin[13] and hydrophobic metabolites[14] from plant-derived samples. Bonded-phase mini-columns are also ideal for the prechromatographic purification of a perbenzoylated sugars,[15] glycopeptides (and derived oligosaccharides),[16] and peralkylated oligosaccharides.[17,18]

**c. Switching Valves, Fittings, and Filters.**—The creative use of switching valves can save chromatographic-run time and extend column life. They can allow elution of the analytical column while the pre-column is being simultaneously cleaned.[12] In chromatograms where they are both very early, and very late peaks, such as those of reducing sugars and their degradation products (for example, furfurals),[19] the use of switching valves can lessen the run time by 50 to 70%.

The myriad types of l.c. fittings produced by a great number of vendors have led to confusion among chromatographers about the proper choice for each application. Fortunately, fittings that are more universal in their applications are now available, and one of the most useful of these has a knurled flange on the nut, and a replaceable, polymeric ferrule. These fittings may be sealed by hand and re-used many times without failure.

The use of in-line filters between injectors and column can prevent the accumulation of particulate material on the inlet frit of an analytical column, and can avoid back-pressure problems. A second, and often overlooked, site for filter installation is between the column and the detector. Cartridge-type filtration-units that contain readily changed, 0.2-$\mu$m filters are commercially available, and they contribute insignificantly to peak broadening. These filters are essential for the prevention of clogged detectors when laboratory-packed columns are used.

**d. Injectors.**—The choice of injector depends upon the particular application, namely, analytical or preparative chromatography. For the former, fixed-loop injectors are far more accurate than the partially filled loop (universal injector) design. For occasional analytical and preparative

(13) G. Bonn, R. Pecina, E. Burtscher, and O. Bobleter, *J. Chromatogr.*, 287 (1984) 215–221.
(14) R. Schwarzenbach, *J. Chromatogr.*, 140 (1977) 304–309.
(15) P. W. Tang and J. M. Williams, *Anal. Biochem.*, 142 (1984) 37–42.
(16) S. J. Swiedler, J. H. Freed, A. L. Tarentino, T. H. Plummer, Jr., and G. W. Hart, *J. Biol. Chem.*, 260 (1985) 4046–4054.
(17) M. W. Spellman, M. McNeil, A. G. Darvill, P. Albersheim, and A. Dell, *Carbohydr. Res.*, 122 (1983) 131–153.
(18) J. M. Lau, M. McNeil, A. G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 137 (1985) 111–125.
(19) D. W. Patrick and W. R. Kracht, *J. Chromatogr.*, 318 (1985) 269–278.

applications, the universal design is useful, because any volume up to a few mL may be injected. For automated, or manual, preparative injection, fixed-loop injectors may be used with large loop sizes (up to 10 or 20 mL) that are currently available. Custom loops are also readily made with 0.5 mm (0.020″) or 0.75 mm (0.030″) (i.d.) tubing, cut to the appropriate length.

**e. Column Design.**—Because the heart of any chromatographic system is the column, considerable attention has been devoted to improvements, through new designs, in its efficiency and stability. The 10-$\mu$m packings and 25-cm-long columns of the 1970's have given way to the 5-$\mu$m (15 cm) and 3-$\mu$m (10 cm) columns available today. These improvements have resulted in faster, higher resolution, and more-sensitive separations and analyses. Although most columns are of the traditional, steel-tube-with-end-fittings design, newer columns are available as cartridges with reusable end-fittings. Such cartridges that fit into dynamic axial compression fittings[8] or radial compression modules[20,21] are especially useful, because voids that develop in the stationary phase are removed by the changing configuration of the module. The diameter of commercial, l.c. columns is also evolving toward wider bores. Initial reports that only long, narrow columns (<5 mm) could produce narrow plate-heights, especially with cation-exchange resin columns,[22] have been disproved,[23] and most commercial columns of that type are now 8 mm wide. Columns that are 30 cm in length, and up to 25 mm in width are also commercially available, and these columns provide resolution as good as, or better[24] than, the narrow (4.0 mm) bore models. These columns are useful for preparative purposes (see preparative section).

**f. Column-packing Equipment.**—Commercially available equipment[25–28] is available for laboratory-packing of analytical and preparative columns. With minimal practice, columns can be packed as efficiently as

(20) J. G. Baust, R. E. Lee, Jr., and H. James, *J. Liq. Chromatogr.*, 5 (1982) 767–779.
(21) N. W. H. Cheetham and V. E. Dube, *J. Chromatogr.*, 262 (1983) 426–430.
(22) M. R. Ladisch and G. T. Tsao, *J. Chromatogr.*, 166 (1978) 85–100.
(23) K. Brunt, *J. Chromatogr.*, 246 (1982) 145–151.
(24) M. Verzele and E. Geeraert, *J. Chromatogr. Sci.*, 18 (1980) 559–570.
(25) J. Kumanotani, R. Oshima, Y. Yamauchi, N. Takai, and Y. Kurosu, *J. Chromatogr.*, 176 (1979) 462–464.
(26) A. D. Jones, I. W. Burns, S. G. Sellings, and J. A. Cox, *J. Chromatogr.*, 144 (1977) 169–180.
(27) G. J. Manius and R. J. Tscherne, *Am. Lab.* (*Fairfield, Conn.*), 13 (1981) 138–145.
(28) S. A. Matlin and L. Chan, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 23–27.

those from commercial sources, and this can generally be done at a small fraction of the cost for a new column. For information on packing a particular phase, see Section II,2. All modern packing is done with slurries of the stationary phase in an appropriate solvent. For silica-based phases, actual packing takes place at pressures of ~28 MPa (4000 lb. $in.^{-2}$) and requires only a few seconds. Cation-exchange resin columns can frequently be packed by gravity sedimentation, followed by further compression with flow from an analytical pump. The packing of columns is one of the most overlooked sources of cost economics in l.c. analyses.

**g. Column Ovens and Post-Column Reaction-Modules.**—Many l.c. columns require elevated temperature for proper performance. Both water jackets and ovens for this purpose are commercially available, but the latter type is more useful and makes leak-detection more practical. Even if an elevated temperature is not required, the enclosure of column, injector, and fittings in a sealed-column compartment (free from sporadic air-currents and temperature changes) markedly improves the baseline on refractive index detector systems. In the absence of column ovens, enclosing the column, tubing, and fittings with large-diameter Tygon tubing will also improve detector stability and sensitivity. Several post-column reaction-modules are commercially available, and many can also serve as column heaters. For details of specific modules, see references listed in Section IV,1d.

**h. Detectors.**—An extensive listing of the characteristics of various l.c. detection systems may be found in Section IV,1. Several general aspects about detectors should be described here, however. In general, the most sensitive detector available for a given separation should be used. Sensitive detection allows injection of very dilute samples and results in greatly extended column-life. Whereas some detector types may be best suited for only a few types of separations, others are more versatile and should be considered first when purchases are made. Photometric detectors that have exchangeable cells for analytical and preparative applications are very useful. Many analytical detectors, especially those of the refractive-index type, have definite limits on flow rates and back pressures, and should not be used for preparative chromatography (flow rates >10 mL/min). One commercially available[8] refractive-index monitor can be used at flow rates of up to 100 mL/min, but it is equally useful for analytical work at <1 mL/min.

**i. Data Systems.**—A number of inexpensive, microprocessor-controlled, recording integrators are now available. These are capable of computing unknown concentrations of carbohydrates by using external or

internal standard methodology on a peak-height or peak-area basis. Although, when used properly, these integrators are ideal for analytical determinations, they should not be used to monitor preparative separations, because they do not operate in "real time," but, rather, with an unspecified, delay time. Traditional strip-chart recorders are best for monitoring proper collection-times for preparative chromatography.

**j. Automated Fraction-Collectors.**—These instruments are exceptionally useful for preparative l.c., especially when they are interfaced, through microprocessors, with the rest of the l.c. system. Certain collectors[7,8] contain so-called "intelligent" modes that permit collection on a peak-sensing mode, as well as on time, drop, or volume modes. These systems are especially useful for automated, preparative chromatography of carbohydrates.[8]

## 2. Stationary Phases

**a. Amine-modified Silica Gels.**—These popular stationary phases are silica gels that have been covalently modified with polar aminopropyl or other amine-containing groups or cyanopropyl-containing groups. The development of these packings in the mid-1970's, an innovation that allowed the development of modern l.c. analysis of sugars, has been thoroughly reviewed.[29,30] Today, amine-modified silica gels are available in a variety of shapes (spherical or irregular) and sizes (3, 5, or 10 $\mu$m) and can be purchased in bulk, or in pre-packed columns, from many commercial suppliers.[29] Moreover, the aminopropyl phase is readily prepared[26] from l.c.-grade silica gel, and can be packed into analytical,[26] or preparative[28]-sized columns with common packing-equipment. For some applications, there is no need to use an amine-bonded silica gel, because, when various amines are added to the mobile phase, ordinary silica-gel columns can be used for sugar separations.[31,32] The advantages and disadvantages of these *in situ* amine-modified columns have been discussed.[9,29]

When amine-modified silica-gel columns are eluted with a mobile phase of acetonitrile–water, carbohydrates are separate on the basis of normal, phase partitioning[33–36]; increasing the water content of the mobile phase

(29) L. A. T. Verhaar and B. F. M. Kuster, *J. Chromatogr.*, 220 (1981) 313–328.
(30) J. F. Pirisino, in J. F. Lawrence (Ed.), *Food Constituents and Food Residues, Their Chromatographic Determination*, Dekker, New York, 1984, pp. 159–193.
(31) K. Aitzetmüller, *J. Chromatogr.*, 156 (1978) 354–358.
(32) B. B. Wheals and P. C. White, *J. Chromatogr.*, 176 (1979) 421–426.
(33) H. Binder, *J. Chromatogr.*, 189 (1980) 414–420.
(34) M. D'Amboise, D. Noël, and T. Hanai, *Carbohydr. Res.*, 79 (1980) 1–10.
(35) L. A. T. Verhaar and B. F. M. Kuster, *J. Chromatogr.*, 234 (1982) 57–64.
(36) Z. L. Nikolov and P. J. Reilly, *J. Chromatogr.*, 325 (1985) 287–293.

speeds up the elution of the sugars. Early investigators mistakenly called these "reversed-phase" columns, and that unfortunate misnomer is still occasionally used. Besides being useful for normal-phase separations, amine-modified silica-gel columns are useful for weak anion-exchange applications when appropriate buffers are used. Details of the proper use of these columns for separation of sugars, oligosaccharides, glycopeptides, and acidic carbohydrates are given in Section III.

Serious attention must be given to the maintenance of aminopropyl-bonded phase columns. The silica gel-based packing will dissolve in water-rich mobile phases, leaving voids in the inlet end. To delay this process, a second column packed with silica gel[10] should be placed between the pump and the injector, to saturate the mobile phase with silica gel. The formation of glycosylamines between reducing sugars and stationary-phase amino groups is another common process that leads to column failure. Certain sugars, such as most pentoses, galactose, mannose, and lactose[10,37,38] readily react with, and deactivate, these stationary-phase amino groups unless the mobile phases are buffered to pH 6 with phosphate buffer.[10] Although it has been claimed[39] that *in situ*, amine-modified silica-gel columns are more durable than covalently bonded types, these columns also dissolve rapidly in their required mobile phases, retention time are quite variable, and, furthermore, they also are not suitable for the analysis of ribose, galactose, arabinose, mannose, and lactose.[9]

When an amino-type column becomes contaminated by physically adsorbed, non-polar materials, it may be cleaned by washing with acetonitrile, hexane, and dichloromethane. If column failure is due to covalent interactions, or to dissolution of the stationary phase, there is very little that can be done to regenerate it. Samples should therefore always be cleaned up, and a pre-column and a saturator column should always be used with the analytical column.

**b. Cation-Exchange Resins.**—During the first half of the 1980's, there was a dramatic increase in the application of resin-based, l.c. columns to the separation of carbohydrates. The mechanisms involved in these separations are not based on newly discovered "ion-moderated partitioning" effects, as many reports have claimed, but are derived from the long-known principles developed by Wheaton and Bauman,[40] Jones and co-

(37) S. R. Abbott, *J. Chromatogr. Sci.*, 18 (1980) 540–550.
(38) C. Brons and C. Olieman, *J. Chromatogr.*, 259 (1983) 79–86.
(39) J. G. Baust, R. E. Lee, Jr., R. R. Rojas, D. L. Hendrix, D. Friday, and H. James, *J. Chromatogr.*, 261 (1983) 65–75.
(40) R. M. Wheaton and W. C. Bauman, *Ann. N.Y. Acad. Sci.*, 57 (1953) 159–176.

workers,[41-43] Barker and associates,[44] and others,[45] for the separation of molecules by "low-pressure" column-chromatographic methods. These techniques were refined over the years (for a review of the chronological development, see Refs. 30 and 46–48), and, today, l.c. columns packed with sulfonated, polystyrene–divinylbenzene spheres of precisely controlled particle sizes (~10 $\mu$m) are routinely used for the separation of every class of carbohydrate listed in Section III, except for the larger oligosaccharides. Pre-packed columns in a variety of particle sizes, ionic forms, and column dimensions are commercially available, and a list of suppliers has been published.[30] Bulk resins, usually in the $H^+$ form, are also available from many of these vendors, and they can be readily converted into the commonly used $Ca^{2+}$ (Refs. 49 and 50) and $Ag^+$ (Ref. 51) forms. The expense of these resins is related to their ze and monodispersity, with the smallest, most precisely sized resins costing the most. Any resin having a particle size of <400 mesh can be used with some success, but those in the 10–15-$\mu$m size give the most efficient analytical and preparative separations. Detailed procedures are available for packing various resins.[49-51] Monodisperse resins pack easily, with the use of only an extra empty column to serve as a reservoir and a union to join the reservoir and packing column together. The soft, 4%-crosslinked resins must be packed with care, to avoid crushing the spheres. The 8%-crosslinked resins are, however, more stable, and can be slurry-packed in water at pressures >14 MPa. Preparative columns[8] (2.2 × 30 cm) are packed with preparative-sized reservoirs, and the larger (30–60 $\mu$m), 4%- and 8%-crosslinked resins should be packed into them at pressures of <7 and 14 MPa, respectively. Small (10 $\mu$m), monodisperse resins are easiest to pack into preparative columns and can be allowed to settle by gravitation before pumping at 3–4 times the normal flow-rate (~10 mL/min).

Hydrogen-form resins are always eluted with dilute, mineral or organic acids (pH 2), and, as such, they are constantly regenerated during use. Hydrochloric acid should not be used, because of its corrosive properties.

(41) J. K. N. Jones, R. A. Wall, and A. O. Pittet, *Chem. Ind. (London)*, (1959) 1196.
(42) J. K. N. Jones, R. A. Wall, and A. O. Pittet, *Can. J. Chem.*, 38 (1960) 2285–2289.
(43) J. K. N. Jones and R. A. Wall, *Can. J. Chem.*, 38 (1960) 2290–2294.
(44) S. A. Barker, B. W. Hatt, J. F. Kennedy, and P. J. Somers, *Carbohydr. Res.*, 9 (1969) 327–334.
(45) S. J. Angyal, G. S. Bethell, and R. J. Beveridge, *Carbohydr. Res.* 73 (1979) 9–18.
(46) R. Wood, L. Cummings, and T. Jupille, *J. Chromatogr. Sci.*, 18 (1980) 551–558.
(47) S. J. Angyal, *Chem. Soc. Rev.*, 9 (1980) 415–428.
(48) K. B. Hicks, P. C. Lim, and M. J. Haas, *J. Chromatogr.*, 319 (1985) 159–171.
(49) M. R. Ladisch, A. L. Huebner, and G. T. Tsao, *J. Chromatogr.*, 147 (1978) 185–193.
(50) L. E. Fitt, W. Hassler, and D. E. Just, *J. Chromatogr.*, 187 (1980) 381–389.
(51) H. D. Scobell and K. M. Brobst, *J. Chromatogr.*, 212 (1981) 51–64.

For $Ca^{2+}$, $Pb^{2+}$, and $Ag^{+}$ columns, pure degassed water is employed as the mobile phase. When used under these conditions, each of these columns has a fairly wide range of applications, as will be discussed in Section III. In general, however, each column is best suited for the following applications: 8%-crosslinked, $H^{+}$ form: sugar acids and alcohols; 4%, $Ca^{2+}$: oligosaccharides (d.p. <6); 8%, $Ca^{2+}$: monosaccharides and sugar alcohols; 4%, $Ag^{+}$: oligosaccharides (d.p. <12–14); and 8%, $Pb^{2+}$: monosaccharides (see Addendum).

The various mechanisms whereby carbohydrates are separated on cation-exchange columns have been the subject of intensive study. In general, a combination of mechanisms, including exclusion, ligand exchange, and hydrophobic adsorption, appears to be involved. The $H^{+}$-form columns,[48] for instance, separate molecules on the basis of ion- and size-exclusion and hydrophobic adsorption, resulting in the elution of carbohydrates of high molecular weight before small ones, acidic carbohydrates before neutral species, and polar sugars before nonpolar (deoxy, acetylated, or methylated) sugar derivatives. The $Ca^{2+}$, $Pb^{2+}$, and $Ag^{+}$ columns separate carbohydrates on the same basis as the $H^{+}$ columns, with the addition of an extra mode, namely, ligand exchange.[52,53] In the last mode, selectivity (and resolution) is brought about by the variable capacity of the hydroxyl groups of each sugar to form complexes with the fixed cation of the stationary phase. Sugar mixtures that cannot be resolved on one ionic form of the resin can often be resolved on another, which will display a different selectivity. Most resin columns should be run at elevated temperatures, because, at room temperature, the partitioning of solutes between the mobile and stationary phases is a diffusion-limited process.[54] Increasing the temperature speeds up the partitioning process, and decreases the height equivalent per theoretical plate. Moreover, at room temperature, mutarotation of most reducing sugars is slow, relative to the chromatographic process, and individual anomers of each sugar are partially resolved, leading to broad peaks. At temperatures of ~85°, mutarotation is faster, and reducing sugars are eluted as one sharp peak. Hence, all $Ca^{2+}$-, $Pb^{2+}$-, and $Ag^{+}$-form columns should be run at 75–85° in order to obtain the highest column-efficiency and resolution. For the separation of sugar acids on $H^{+}$-form resins, however, high temperature should usually be avoided, because of partial, or complete, conversion of these acids, *in situ*, into various lactone forms.[48]

(52) S. J. Angyal, *Tetrahedron*, 30 (1974) 1695–1702.
(53) R. W. Goulding, *J. Chromatogr.*, 103 (1975) 229–239.
(54) R. Pecina, G. Bonn, E. Burtscher, and O. Bobleter, *J. Chromatogr.*, 287 (1984) 245–258.

Cation-exchange resin columns are relatively easy to maintain, and this is a major reason for their popularity. Unlike silica-based columns, they are not sensitive to extreme pH values, and they do not dissolve in aqueous, mobile phases. As in the case of all l.c. columns, the key to long life is effective, sample clean-up. Samples should, whenever possible, be pretreated with small amounts of cation-exchange resin in the appropriate ionic form, and pre-columns containing a similar resin in the same ionic form should be used, and changed regularly. Inexpensive, cation-exchange resins (minus 400-mesh size) can be slurry-packed into pre-columns, and their use adds very little to peak broadening. Mixed-bed resins[54a] can be used in pre-columns to de-ash samples. When this is done, the anion-exchange resin must be of a weak-base variety, because strong-base resins absorb sugars.[55,56] Convenient, cartridge-type pre-columns are also commercially available. Various mobile-phase additives, such as calcium acetate[57] and calcium ethylenediaminetetraacetate[23] have been used in order to allow constant regeneration of the $Ca^{2+}$-form columns. This, of course, adds another step to mobile-phase preparation and is unsuitable for preparative use. With proper sample clean-up, use of such additives should be unnecessary. If, however, a column becomes contaminated, the back-pressure will rise to unacceptable limits. If this is due to non-polar materials that have adsorbed to the packing, the column should be backflushed with 3 : 7 acetonitrile–water. If column failure is due to ionically bound species, it must be emptied, and the resin must be cleaned by treatment with 3 *N* HCl, converted into the desired ionic form, and repacked as already described. The regeneration of the phase inside the column is not recommended.

**c. Alkylated (Reversed-Phase) Silica Gels.**—These durable phases are extremely useful for the analysis and preparation of less-polar carbohydrates and such carbohydrate derivatives as those encountered in the synthesis laboratory. Quite often, complex mixtures such as these may be readily fractionated on a 15- to 25-cm-long column packed with 3-$\mu$m size $C_{18}$-bonded silica gel, using only pure water, or dilute methanol, as the isocratic eluant. A good example of this, covered in detail in a later Section, is in the separation of isomeric glycosides formed during the reaction of reducing sugars or sugar acids with methanol in the presence of an acid catalyst. Often, reactions can be more effectively examined for

(54a) P. L. Keeling and P. James, *J. Liq. Chromatogr.*, 9 (1986) 983–992.
(55) M. Tanaka, *Carbohydr. Res.*, 88(1981) 1–8.
(56) M. L. Richmond, D. L. Barfuss, B. R. Harte, J. I. Gray, and C. M. Stine, *J. Dairy Sci.*, 65 (1982) 1394–1400.
(57) D. C. Woollard, *N. Z. J. Dairy Sci. Technol.*, 18 (1983) 209–224.

particular products by this method than by t.l.c., as in the analysis of synthetic mixtures containing diastereoisomeric pyrimidine nucleoside antibiotics[57a] and isomeric 2′-deoxy-*C*-nucleosides.[57b] For more-difficult separations, such as the separation of diastereoisomeric guanosine derivatives[57c] and coformycin analogs,[57d] simple binary gradients, composed of water (or phosphate buffer) and methanol, provide good separations. Other so-called "reversed-phase" silica-gel phases may have better selectivities for particular separations than the common $C_{18}$ variety. A "phenyl"-bonded phase, for instance, was found useful for the analysis of synthetic reactions containing adriamycin analogs.[57e] Although these methods are quite useful, they do not replace t.l.c. for the routine monitoring of reactions. In most cases, the greater expense of l.c., combined with its lower speed make it an impractical method for monitoring reactions, especially as many reactions can be monitored simultaneously on one t.l.c. plate. However, when quantitative results are desired, or when separations of similar compounds cannot be achieved by t.l.c., such l.c. methods as these are advised. The use of l.c. as a preparative method will be covered later, but it is relevant to mention here that, for nonpolar carbohydrate derivatives, reversed-phase type silica gels also function quite well in this capacity. Various nucleoside derivatives,[57a,57d] for instance, have been isolated on preparative-sized (2.2 × 50 cm) columns packed with $C_{18}$ silica gels. Because of the low capacities of columns of these types (maximum of 100–150 mg per injection), they will not soon replace the more-common types of silica-gel chromatography or their "flash"-type modifications, for the isolation of large quantities of reaction products. The same, reversed-phase, silica-gel columns that are used for 80–90% of all other l.c. applications find only limited use in analysis of polar carbohydrates. This is primarily due to the poor retention of these polar, water-soluble compounds on the nonpolar, alkylated, stationary phases when even the weakest mobile phase, namely, water, is used. Nevertheless, for several carbohydrate applications,[57b,g] these columns are ideal, and therefore deserve consideration.

(57a) J. C. Boehm and W. D. Kingsbury, *J. Org. Chem.*, 51 (1986) 2307–2314.
(57b) J. C.-Y. Cheng, U. Hacksell, and G. D. Daves, Jr., *J. Org. Chem.*, 51 (1986) 3093–3098.
(57c) R. C. Moschel, K. Hemminki, and A. Dipple, *J. Org. Chem.*, 51 (1986) 2952–2955.
(57d) O. L. Acevedo, S. H. Krawczyk, and L. B. Townsend, *J. Org. Chem.*, 51 (1986) 1050–1058.
(57e) N. Kumar, R. Seshadri, and M. Israel, *Carbohydr. Res.*, 153 (1986) 171–180.
(57f) E. Rajakylä, *J. Chromatogr.*, 353 (1986) 1–12.
(57g) G. D. McGinnis, S. Prince, and J. Lowrimore, *J. Carbohydr. Chem.*, 5 (1986) 83–97.

When water is used as the mobile phase, $C_{18}$-bonded silica-gel columns are quite useful for the analysis of the more nonpolar or higher-molecular-weight carbohydrates, such as methyl glycosides,[58] chitin-[59] or starch[60]-derived oligosaccharides, and such *N*-acetylated or deoxy sugar-containing oligosaccharides as those in human milk[61] and glycopeptides.[62] For analysis of very nonpolar derivatives, such as peralkylated oligosaccharides,[63] or many of the pre-column l.c. derivatives discussed in Section IV,1, such organic modifiers as acetonitrile or primary alcohols[64] must be added to the mobile phase to shorten retention times. Conversely, some success has been achieved in enhancing the retention of normally unretained sugars by addition of salts to the mobile phase.[64,65] When such hydrophobic bases as alkylamines are added to the aqueous mobile phase, such acidic carbohydrate derivatives as ascorbic acids,[66] uronates,[67] and sulfated oligosaccharides[68] are retained and separated by ion-pairing mechanisms.

A major disadvantage of reversed-phase columns is that they resolve the anomeric forms of reducing sugars, and this leads to broad, complex peaks. Neither the addition of basic mutarotation catalysts, nor elevation of column temperature can satisfactorily correct this problem without destroying the column, or decreasing the resolution, respectively.[65] However, nonreducing sugars and those oligosaccharides having chemically reduced, reducing ends, produce sharp peaks on these columns. Alkylated silica-gel columns have low sample-capacity and are therefore of limited use in preparative applications.

Reversed-phases are generally very stable, and do not dissolve in aqueous mobile phases, as do normal-phase silica-gel columns. After injection of many samples that contain highly retained, nonpolar materials, resolu-

(58) N. W. H. Cheetham and G. Teng, *Carbohydr. Res.*, 144 (1985) 169–175.
(59) K. Blumberg, F. Liniere, L. Pustilnik, and C. A. Bush, *Anal. Biochem.*, 119 (1982) 407–412.
(60) N. W. H. Cheetham, P. Sirimanne, and W. R. Day, *J. Chromatogr.*, 207 (1981) 439–444.
(61) V. K. Dua and C. A. Bush, *Anal. Biochem.*, 133 (1983) 1–8.
(62) V. K. Dua and C. A. Bush, *Anal. Biochem.*, 137 (1984) 33–40.
(63) M. McNeil, A. G. Darvill, P. Åman, L.-E. Franzén, and P. Albersheim, *Methods Enzymol.*, 83 (1982) 3–45.
(64) L. A. T. Verhaar, B. F. M. Kuster, and H. A. Claessens, *J. Chromatogr.*, 284 (1984) 1–11.
(65) N. W. H. Cheetham and G. Teng, *J. Chromatogr.*, 336 (1984) 161–172.
(66) C. S. Tsao and M. Young, *J. Chromatogr.*, 330 (1985) 408–411.
(67) A. G. J. Voragen, H. A. Schols, J. A. De Vries, and W. Pilnik, *J. Chromatogr.*, 244 (1982) 327–336.
(68) A. Heyraud and C. Rochas, *J. Liq. Chromatogr.*, 5 (1982) 403–412.

tion may decrease, but this can generally be corrected by washing the column with methanol and boric acid solutions.[69] Methods which detail the procedures for packing these columns have been published.[27]

**d. Anion-Exchange Resins and Silica Gels.**—Both silica-gel- and polystyrene-based, anion-exchange, l.c. stationary-phases are commonly used for the separation of the many classes of acidic carbohydrates listed in Section III. These packings, which bear quaternary ammonium groups, are available in efficient 5- and 10-$\mu$m particle sizes. The development of modern anion-exchange phases, their various physical characteristics, and commercial suppliers have been reviewed.[46] Resin-type and silica-based anion-exchange particles can be packed into columns by using the methods described earlier for cation-exchangers and aminopropyl silica gels, respectively, and by following manufacturer's directions. These columns are often eluted with phosphate buffers, or salt solutions, for separation of simple and complex acidic carbohydrates. When boric acid-containing buffers are employed,[70,71] neutral as well as acidic sugars can be separated by borate-complexation mechanisms. Unfortunately, these separations are rather lengthy (>1h).

Silica-based anion-exchangers tend to have short life-times. This may be extended somewhat by proper sample clean-up and by the use of a silica-gel-saturation column, but this rapid degradation remains the biggest disadvantage of these phases. The resin-based phases are very stable, unless operated above 65°. Unfortunately, because of the slow diffusion processes in these resins, they must be operated at higher temperatures in order to achieve good efficiencies.

A unique anion-exchange column has been developed[72] that has a thin (non-diffusion limited) anion-exchange phase coated onto a 10-$\mu$m latex bead. When a mobile phase of 0.15 *M* NaOH is used, neutral carbohydrates are converted into anions, which are separated on the column. Although the resin has low capacity, and probably causes degradation of the carbohydrates, when it is coupled to a triple-pulsed, amperometric detector, the system provides extremely sensitive, high-resolution separations.

(69) P. Vrátný, J. Čoupek, S. Vozka, and Z. Hostomská, *J. Chromatogr.*, 254 (1983) 143–155.
(70) S. Honda, M. Takahashi, K. Kakehi, and S. Ganno, *Anal. Biochem.*, 113 (1981) 130–138.
(71) S. Honda, S. Suzuki, M. Takahashi, K. Kakehi, and S. Ganno, *Anal. Biochem.*, 134 (1983) 34–39.
(72) R. D. Rocklin and C. A. Pohl, *J. Liq. Chromatogr.*, 6 (1983) 1577–1590.

**e. Miscellaneous Stationary Phases.**—Several less well-known, stationary phases have been used for carbohydrate separations. Boronic acid-substituted silica gel,[73,74] for example, is useful for chromatography of sugars and nucleosides that contain *cis*-diols. When copper(II) silicate gel is used as the stationary phase,[75,76] sugars are separated by combined ligand-exchange and normal phase-partition modes. This phase can be readily synthesized by treating l.c.-grade silica gel with ammoniated copper sulfate solutions. Another useful silica derivative, diol-modified silica gel,[38] appears to function like aminopropyl silica gel, but it is much more robust, and can be used for separation of pentoses and hexoses that cannot be analyzed on the amine-modified phases. A similar phase, a so-called "polyol" bonded silica gel,[76a] has also been used to separate various mono-, di-, and tri-saccharides. As on the previously mentioned diol phase, however, reducing sugars are eluted from this polyol phase in broad, or double, peaks (resolution of anomeric forms). Such mutarotation catalysts as triethylamine can be added to the mobile phase to produce sharp, symmetrical peaks, but this practice is usually destructive to these silica gel-based packings.

Although ion-exchange resins[77] have often been used for normal-phase, partition chromatography in open, glass columns, this technique has not been easily adapted to high-performance and high-pressure applications. However, new resins have now been developed that are of the correct size (5 $\mu$m) and rigidity (>50% cross-linking with divinylbenzene) to withstand high pressures and the high flow-rates of acetonitrile–water mobile phases. The application of these resins for the separation of monosaccharides, deoxy sugars, 2-acetamido-2-deoxy-D-hexoses, and sialic acids has been demonstrated.[78–80a] It is anticipated that the latter stationary phases will be used much more frequently in the future.

(73) M. Glad, S. Ohlson, L. Hansson, M.-O. Månsson, and K. Mosbach, *J. Chromatogr.*, 200 (1980) 254–260.
(74) E. Hagemeier, K.-S. Boos, E. Schlimme, K. Lechtenbörger, and A. Kettrup, *J. Chromatogr.*, 268 (1983) 291–295.
(75) J. L. Leonard, F. Guyon, and P. Fabiani, *Chromatographia*, 18 (1984) 600–602.
(76) F. Guyon, A. Foucault, M. Caude, and R. Rosset, *Carbohydr. Res.*, 140 (1985) 135–138.
(76a) M. Verzele and F. Van Damme, *J. Chromatogr.*, 362 (1986) 23–31.
(77) O. Samuelson, *Adv. Chromatogr.*, 16 (1978) 113–149.
(78) S. Honda, S. Suzuki, and K. Kakehi, *J. Chromatogr.*, 291 (1984) 317–325.
(79) S. Honda, T. Konishi, and S. Suzuki, *J. Chromatogr.*, 299 (1984) 245–251.
(80) S. Honda and S. Suzuki, *Anal. Biochem.*, 142 (1984) 167–174.
(80a) A. Sugii, K. Harada, and Y. Tomita, *J. Chromatogr.*, 366 (1986) 412–416.

## III. Separations and Applications

### 1. Analytical Separations

**a. Neutral Mono- and Di-saccharides.**—A great number of the published l.c. methods for carbohydrates deal with the simple mono- and di-saccharides, and this fact is verified by the many comprehensive reviews that cover this subject.[1,2,29,81–82a] As such, the reader is referred to those reviews, which contain details of specific separations of simple and complex mixtures.

As has been pointed out,[81] no general conditions and equipment are currently available for the simultaneous l.c. separation of all of the known monosaccharides. Fortunately, however, this necessity seldom arises, and, instead, methods are more often needed for the separation of the mono- and di-saccharides present in such samples as foods, pharmaceuticals, and hydrolyzates of polysaccharides, glycoconjugates, and "biomass" materials. Specific procedures for these examples are given in Section III,2. Additional key references that describe advance in the separation of mono- and di-saccharides are given in Table I. In general, the method selected for a given analysis of sugars should be the one that provides the simplest and fastest separation, without sacrificing accuracy of analysis or column-life.

Because cation-exchange resin-based columns ($Ca^{2+}$ or $Pb^{2+}$ form) provide convenient, accurate, rapid, reproducible, and sensitive analyses, and use only water as the mobile phase, they are generally the method of choice, despite some claims to the contrary.[39] Most mixtures of monosaccharides can be resolved on one or the other form of these columns (see Ref. 30 for a general comparison). However, for certain other applications, such as the simultaneous analysis of several disaccharides, amine-modified silica-gel columns must be used,[39,90] despite their relative instability and requirement for a highly pure, acetonitrile-containing, mobile phase. Reversed-phase silica-gel columns are of little use for separation of polar mono- and di-saccharides, because of their low capacity factors. More-nonpolar sugar derivatives, such as 2-acetamido-2-deoxy derivatives, are retained on $C_{18}$-bonded silica gel,[59] but are separated into two, or more, broad (difficult to analyze) anomeric peaks. Such columns are very useful for separation of simple glycosides.[5,91,92] A column used for monosaccharide separation[80] combines the stability of ion-exchange resins with the high resolving power of normal, phase-partitioning using acetonitrile–water as the mobile phase, and further use of this method is expected.

(81) S. Honda, *Anal. Biochem.*, 140 (1984) 1–47.
(82) A. Heyraud and M. Rinaudo, *J. Liq. Chromatogr.*, 4 (Suppl. 2) (1981) 175–293.
(82a) K. Robards and M. Whitelaw, *J. Chromatogr.*, 373 (1986) 81–110.

TABLE I

**Selected L. C. Methods for the Analysis of Mono- and Di-saccharides**

| Class | Members |
|---|---|
| Aldoses | allose and altrose,[39] arabinose,[10,39,83] galactose and glucose,[38,39,83] ribose,[39] mannose and xylose[10,39,83] |
| Ketoses | fructose,[38,39,84] psicose,[84] *erythro*-2-pentulose and sorbose,[33,39] tagatose[39,85] |
| Alditols | arabinitol, galactitol, ribitol, mannitol, and glucitol,[38,39,46,86] xylitol[19,39,86] |
| Deoxy sugars | 2-deoxy-*arabino*-hexose,[39,87] 6-deoxyglucose and 2-deoxy-*erythro*-pentose,[39] fucose and rhamnose,[39,80] 2-acetamido-2-deoxyhexoses[59,80,88] |
| Disaccharides | cellobiose and cellobiulose,[39,89,90] (gentiobiose, isomaltose, kojibiose, laminarabiose, leucrose, melibiose, nigerose, palatinose, sophorose, trehalose, turanose, and xylobiose),[39,90] lactose and lactulose,[38,85] maltose and maltulose[38,89] |

**b. Ionic Mono- and Di-saccharides.**—Common members in this category are the uronic, aldonic, keto-aldonic, and aldaric acids, which occur in metabolic pathways, biopolymers, and fermentative processes. For analysis of simple mixtures of uronic acids, methods based on ion-exclusion chromatography[25,48,93] are rapid (<15 min) and accurate. When several uronic acids exist in the same sample, separations are best performed on resin-[71,94,95] or silica-based,[67,96] strong anion-exchange columns, which provide greater resolution. The silica-based columns are somewhat unsta-

(83) R. C. Pettersen, V. H. Schwandt, and M. J. Effland, *J. Chromatogr. Sci.*, 22 (1984) 478–484.
(84) L. W. Doner, *Carbohydr. Res.*, 70 (1979) 209–216.
(85) K. B. Hicks, D. L. Raupp, and P. W. Smith, *J. Agric. Food Chem.*, 32 (1984) 288–292.
(86) S. Honda, M. Takahashi, S. Shimada, K. Kakehi, and S. Ganno, *Anal. Biochem.*, 128 (1983) 429–437.
(87) D. E. Hughes, *J. Chromatogr.*, 331 (1985) 183–186.
(88) W. M. Blanken, M. L. E. Bergh, P. L. Koppen, and D. H. van den Eijnden, *Anal. Biochem.*, 145 (1985) 322–330.
(89) K. B. Hicks, E. V. Symanski, and P. E. Pfeffer, *Carbohydr. Res.*, 112 (1983) 37–50.
(90) Z. L. Nikolov, M. M. Meagher, and P. J. Reilly, *J. Chromatogr.*, 319 (1985) 51–57.
(91) N. W. H. Cheetham and P. Sirimanne, *J. Chromatogr.*, 208 (1981) 100–103.
(92) A. Hjerpe, B. Engfeldt, T. Tsegenidis, and C. A. Antonopoulos, *J. Chromatogr.*, 259 (1983) 334–337.
(93) R. Oshima, Y. Kurosu, and J. Kumanotani, *J. Chromatogr.*, 179 (1979) 376–380.
(94) K. Mopper, *Anal. Biochem.*, 86 (1978) 597–601.
(95) M. J. Spiro, *Anal. Biochem.*, 82 (1977) 348–352.
(96) P. Gacesa, A. Squire, and P. J. Winterburn, *Carbohydr. Res.*, 118 (1983) 1–8.

ble, but provide rapid, simultaneous separations of galacturonic, mannuronic, and glucuronic acids.[67] For the separation of the *D*-mannuronic and *L*-guluronic acids that exist in hydrolyzates of alginates, l.c. on strong anion-exchange silica-gel columns has been recommended.[67,96] The polystyrene-based resins are more stable, but are not useful for rapid separations. Often as long as 60 min is required to resolve such complex mixtures as that of iduronic, glucuronic, mannuronic, and galacturonic acids.[71]

Surprisingly few l.c. methods are yet available for the aldonic acids. These acids have been separated from non-acidic compounds by chromatography on strong[97–99] and on weak[100,101] anion-exchangers. Separations of ribonic, D-*glycero*-D-*gulo*-heptonic, mannonic, gluconic, and galactonic acids have been successful on strong cation-exchange resins.[48,101,102]

Because of their involvement in several economically important fermentation pathways, several methods have been developed to separate the aldulosonic acids. During the biotechnological conversion of D-glucose into D-fructose by way of D-*arabino*-hexos-2-ulose (D-glucosone),[100] the levels of the by-products D-gluconic (**1**) and D-*arabino*-2-hexulosonic (**2**) acids were rapidly (<15 min) measured by l.c. on an aminopropyl silica-gel column. Similar separations are possible on cation-exchange resin, l.c. columns,[48] which are more durable and economical to use. These columns have also been used for more-complex mixtures, such as those products and by-products from the fermentation of L-sorbose during L-ascorbic acid synthesis,[102] namely, **1, 2**, L-*xylo*-2-hexulosonic (**3**), and D-*xylo*-5-hexulosonic (**4**) acids, and D-glucitol. However, with this system acids **2** and **3** are separated poorly, as are D-gluconic acid (**1**) and L-sorbose. For analysis of mixtures of acids **2–4** and 2,5-D-*threo*-hexodiulosonic acid (**5**), such as those produced by genetically engineered bacteria,[103] or acids **1, 2, 4,** and **5** (produced by common soil-bacteria),[98] polystyrene-based, strong anion-exchange resin columns, eluted with buffers containing formic acid or formic salts, are recommended.

Separation of aldaric acids on anion-exchange,[97] aminopropyl silica

(97) J. M. H. Dirkx and L. A. T. Verhaar, *Carbohydr. Res.*, 73 (1979) 287–292.
(98) J. D. Blake, M. L. Clarke, and G. N. Richards, *J. Chromatogr.*, 312 (1984) 211–219.
(99) P. J. M. Dijkgraaf, L. A. T. Verhaar, W. P. T. Groenland, and K. van der Wiele, *J. Chromatogr.*, 329 (1985) 371–378.
(100) J. Geigert, D. S. Hirano, and S. L. Neidleman, *J. Chromatogr.*, 202 (1980) 319–322.
(101) E. Rajakylä, *J. Chromatogr.*, 218 (1981) 695–701.
(102) A. W. Anderson and G. T. Tsao, *Biotechnol. Bioeng.*, 26 (1984) 374–376.
(103) S. Anderson, C. Marks, R. Lazarus, J. Miller, K. Stafford, J. Seymour, D. Light, W. Rastetter, and D. Estell, *Science*, 230 (1985) 144–149.

gel,[104] and strong anion-exchange silica gel[105] is difficult, because these acids are eluted in broad peaks. Addition of magnesium ions to the mobile phase tends to speed up the elution of aldaric acids, and helps to provide somewhat sharper peak-geometries.[99] Peak broadening is partially due to the coexistence of acid and lactone forms, and this effect must be taken into account whenever sugar acids are chromatographed. In fact, many sugar acids can be baseline-separated from their lactone forms on amino-propyl silica gel,[100,101,104] anion exchange,[98] and cation-exchange[48] columns, allowing the simultaneous determination of each form of the compound in a single sample. If individual levels of acid and lactone are not of interest, all lactones should be converted into the acid form prior to chromatography.[98]

Methods for the l.c. analysis of ascorbic acids and related compounds in foods, pharmaceuticals, and physiological tissues have been extensively reviewed elsewhere.[106,107] Ascorbic acid may be determined after separation on cation-exchange l.c. columns ($H^+$ form),[108] but care must be taken to prevent on-column degradation.[48] This system only marginally separates L-ascorbic acid from its C-5 epimer, isoascorbic acid. Although ascorbic acids have little retention on reversed-phase silica gel, the use of ion-pairing reagents improves their retention and allows the separation of ascorbic and isoascorbic acids.[109] Ascorbic acid and its oxidation product, dehydroascorbic acid, have been separated on reversed-phase[110] and on hydrophilic gel columns.[111] Both strongly and weakly basic anion-exchangers have been used for ascorbic acid analysis,[107] and the latter[112] system has been used to separate, completely and rapidly (<15 min), ascorbic, isoascorbic, dehydroascorbic, dehydroisoascorbic, and *threo*- and *erythro*-2,3-hexodiulosonic acids. The 2-sulfate and 2-phosphate of L-ascorbic acid are of biomedical importance, and their separations have

(104) E. I. Laakso, R. A. Tokola, and E. L. Hirvisalo, *J. Chromatogr.*, 278 (1983) 406–411.
(105) D. G. Walters, B. G. Lake, D. Bayley, and R. C. Cottrell, *J. Chromatogr.*, 276 (1983) 163–168.
(106) L. A. Pachla, D. L. Reynolds, and P. T. Kissinger, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 1–12.
(107) L. W. Doner, in J. Lawrence (Ed.), *Trace Analysis*, Vol. 3, Academic Press, New York, 1984, pp. 113–138.
(108) M. Grün and F. Loewus, *Anal. Biochem.*, 130 (1983) 191–198.
(109) C. S. Tsao and S. L. Salimi, *J. Chromatogr.*, 245 (1982) 355–358.
(110) J. W. Finley and E. Duang, *J. Chromatogr.*, 207 (1981) 449–453.
(111) T. Seki, Y. Yamaguchi, K. Noguchi, and Y. Yanagihara, *J. Chromatogr.*, 332 (1985) 283–286.
(112) L. W. Doner and K. B. Hicks, *Anal. Biochem.*, 115 (1981) 225–230.

also been noted.[113] An impressive separation of ascorbic acid, 2-*O*-methylascorbic acid, 3,4-dihydroxy-5-methyl tetrone, ascorbic acid 2-phosphate, ascorbic acid 2-sulfate, and 6-bromo-6-deoxy-L-ascorbic acid was achieved in less than 15 min on a reversed-phase–ion-pairing system.[66]

Relatively few methods exist for the l.c. separation of sugar phosphates. Simple mixtures containing a sugar phosphate and a neutral aldose can be resolved by anion-[114] or cation-exchange columns.[115] Glucosyl phosphate and glucose 6-phosphate have been separated on an ion-pairing, reversed-phase system,[116] and some low-resolution separations of pentose, hexose, and heptose mono- and di-phosphates have been achieved on strong anion-exchange silica gel.[117,118] The isomeric sugar phsopates, D-fructose 6- and D-glucose 6-phosphate, have been separated by ion-pairing, reversed phase l.c.[118a] A variation[118b] of the same system was used to separate inositol tri-, tetra-, penta-, and hexa-phosphates.

Various deoxy-2-octulosonic acids [for example, 3-deoxy-D-*manno*-2-octulosonic acid, (KDO)], neuraminic acids, and their derivatives have been chromatographed on l.c. columns packed with anion-exchange resins,[119–122] cation-exchange resins,[123] amine-modified silica gels,[124,125] and reversed-phase silica gels.[124] Although class separations of neutral sugars from these acidic compounds may be obtained on cation-exchangers, very little separation of individual sialic acids occurs. KDO and several derivatives of neuraminic acid have been more successfully separated on

(113) D. Mauro, D. Wetzel, C. H. Lee, and P. A. Seib, *J. Chromatogr.*, 187 (1980) 421–428.
(114) K. Brunt and H. Hokse, *J. Chromatogr.*, 268 (1983) 131–137.
(115) R. M. Stikkelman, T. T. Tjioe, J. P. van der Wiel, and F. Van Rantwijk, *J. Chromatogr.*, 322 (1985) 220–222.
(116) T. T. Tjioe, J. P. van der Wiel, R. M. Stikkelman, A. J. J. Straathof, and F. Van Rantwijk, *J. Chromatogr.*, 330 (1985) 412–414.
(117) C. Giersch, *J. Chromatogr.*, 172 (1979), 153–161.
(118) S. K. Henderson and D. E. Henderson, *J. Chromatogr. Sci.*, 23 (1985) 222–226.
(118a) S. K. Henderson and D. E. Henderson, *J. Chromatogr. Sci.*, 24 (1986) 198–203.
(118b) A.-S. Sandberg and R. Ahderinne, *J. Food Sci.*, 51 (1986) 547–550.
(119) A. K. Shukla, N. Scholz, E. H. Reimerdes, and R. Schauer, *Anal Biochem.*, 123 (1982) 78–82.
(120) A. K. Shukla and R. Schauer, *J. Chromatogr.*, 244 (1982) 81–89.
(121) A. K. Shukla, R. Schauer, U. Schade, H. Moll, and E. T. Rietschel, *J. Chromatogr.*, 337 (1985) 231–238.
(122) A. K. Shukla, R. Schauer, F. M. Unger, U. Zähringer, E. T. Rietschel, and H. Brade, *Carbohydr. Res.*, 140 (1985) 1–8.
(123) H. K. B. Silver, K. A. Karim, M. J. Gray, and F. A. Salinas, *J. Chromatogr.*, 224 (1981) 381–388.
(124) S. Diaz and A. Varki, *Anal. Biochem.*, 150 (1985) 32–46.
(125) H. Fiedler and H. Faillard, *Chromatographia*, 20 (1985) 231–234.

polystyrene-based, anion-exchange resins eluted with formate,[121] sulfate[120,122] or borate buffer.[119] On these columns, retention is affected by the number of acetyl groups present on the neuraminic acid derivative; consequently, mono-, di-, and tri-*O*-acetylated acids are readily separated, as are many of the *N*-acetyl and *N*-glycolyl derivatives. When borate is included in the mobile phase, separations are also affected by the ability of the sialic acids to form stable borate complexes, and this leads to orders of elution different from those produced with formate or sulfate buffers. The more highly *O*-acetylated derivatives are also well separated on reversed-phase columns by using an ion-pairing mode.[124] By application of one or more of these methods, mixtures of sialic acids most commonly encountered can be resolved, except for positional isomers.

Sulfated disaccharides, such as those resulting from the breakdown of the chondroitin sulfates, have been separated on aminocyanopropyl silica gel,[126] aminopropyl silica gel,[126,127] and cation-exchange columns.[128,129] Each of these columns produces distinctly different orders of elution, allowing the ready identification of chondroitin disaccharides by chromatography of unknowns *versus* standards on two or three of the different phases. Oversulfated chondroitin disaccharides, containing 2 or 3 sulfate groups, may also be resolved.[128,130,131] Similarly, sulfated disaccharides from enzyme- or nitrous acid-treated heparin have been separated on reversed-phase,[132] polar aminocyanopropyl silica gel,[132] and strong anion-exchange silica gel columns.[133] Often, these disaccharides are reduced with sodium borotritide prior to chromatography; this results in better chromatographic resolution and the ability to use sensitive, radiochemical-detection methods.

**c. Simple, Neutral Oligosaccharides.**—Within the area of oligosaccharide separations, the largest variety of l.c. methods has been developed for the $\alpha$-(1 $\rightarrow$ 4)-linked D-glucose oligosaccharides (malto-oligosaccharides), such as those present in starch hydrolyzates. Effective separations have been achieved on columns packed with aminopropyl-

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(127) A. Hjerpe, C. A. Antonopoulos, and B. Engfeldt, *J. Chromatogr.*, 171 (1979) 339–344.
(128) K. Murata and Y. Yokoyama, *Anal. Biochem.*, 146 (1985) 327–335.
(129) K. Murata and Y. Yokoyama, *Anal. Biochem.*, 149 (1985) 261-268.
(130) A. Hjerpe, C. A. Antonopoulos, B. Engfeldt, and M. Nurminen, *J. Chromatogr.*, 242 (1982) 193–195.
(131) D. C. Seldin, N. Seno, K. F. Austen, and R. L. Stevens, *Anal. Biochem.*, 141 (1984) 291–300.
(132) G. J.-L. Lee and H. Tieckelmann, *J. Chromatogr.*, 195 (1980) 402–406.
(133) S. R. Delaney, M. Leger, and H. E. Conrad, *Anal. Biochem.*, 106 (1980) 253–261.

bonded silica gel,[134–137] aminocyanopropyl-bonded silica gel,[138] *in situ* amine-modified silica gel,[139,140] pure silica gel,[141] conventional[60,64,65,69,142] and aminopropyl-"doped,"[143] $C_{18}$-bonded, reversed-phase silica gel, polystyrene-based anion-exchangers used in either a normal phase-partition,[144] or ion-chromatographic[72] mode, or cation-exchange resins used in $Ca^{2+}$ (Refs. 23 and 50) or $Ag^{+}$ (Refs. 51 and 145) forms. Aminopropyl and aminocyanopropyl silica gel columns both allow the rapid (d.p. 1–15 in 15 min) separation of these oligosaccharides in ascending order when aqueous acetonitrile is used as the mobile phase. The limited solubility of higher-d.p. oligosaccharides, dextrins, and starch in this mobile phase may, however, cause peak broadening, sample precipitation, and eventual fouling of the packing, unless samples are pretreated to remove these insoluble components. Moreover, glycosylamine formation between reducing oligosaccharides and stationary-phase aminopropyl groups[10,37] can also lead to column failure (see Section II,2a).

Pure silica gel columns that are physically modified *in situ* with aliphatic amines appear to be more robust in such applications, as the silica-absorbed amine is constantly regenerated. Use of such a stationary phase[140] allowed the separation of malto-oligosaccharides from d.p. 2 to 23 in 40 min. Reversed-phase ($C_{18}$) packings (with pure water as the mobile phase) are also quite durable in these applications, but, unfortunately, the anomeric forms of reducing oligosaccharides are partially resolved on these columns, leading to broad and difficult-to-analyze peaks. Although no practical solvent-modifier or temperature effect has been found to prevent this peak doubling, a chemical modification (aminopropyl doping)[143] of $C_{18}$ columns has been used to eliminate this effect. At present, this column appears to be ideal for efficient, reproducible, and economical separation of $\alpha$-(1 → 4)-linked gluco-oligosaccharides. Cation-exchange resins, which also may be eluted with pure water as the mobile phase, separate

(134) R. Schwarzenbach, *J. Chromatogr.*, 117 (1976) 206–210.
(135) V. Kahle and K. Tesarík, *J. Chromatogr.*, 191 (1980) 121–128.
(136) K. Kainuma, T. Nakakuki, and T. Ogawa, *J. Chromatogr.*, 212 (1981) 126–131.
(137) Ž. L. Nikolov, J. B. Jakovljević, Ž. M. Boškov, and N. Sad, *Starch/Staerke*, 36 (1984) 97–100.
(138) F. M. Rabel, A. G. Caputo, and E. T. Butts, *J. Chromatogr.*, 126 (1976) 731–740.
(139) K. Aitzetmüller, *Chromatographia*, 13 (1980) 432–436.
(140) C. A. White, P. H. Corran, and J. F. Kennedy, *Carbohydr. Res.*, 87 (1980) 165–173.
(141) S. Iwata, T. Narui, K. Takahashi, and S. Shibata, *Carbohydr. Res.*, 133 (1984) 157–162.
(142) A. Heyraud and M. Rinaudo, *J. Liq. Chromatogr.*, 3 (1980) 721–739.
(143) B. Porsch, *J. Chromatogr.*, 320 (1985) 408–413.
(144) J. Havlicek and O. Samuelson, *Anal. Chem.*, 47 (1975) 1854–1857.
(145) J. J. Warthesen, *Cereal Chem.*, 61 (1984) 194–195.

oligosaccharides in the order of descending molecular weight, owing to size-exclusion effects.[44,50] Resins with crosslinking percentages between 4 and 8%, in the $Ca^{2+}$ or $Ag^{+}$ form, may be used as stationary phases according to the following general rules: resins with low percentage of crosslinking are less mechanically stable but allow resolution of saccharides of higher d.p. value. Silver-form resins are more mechanically stable than resins in the $Ca^{2+}$ form, and they also allow resolution of higher-d.p. saccharides. All cation-exchange resin ($Ca^{2+}$ and $Ag^{+}$ form) columns must be operated at temperatures of 75–85° to produce optimum resolution. For high-conversion corn syrups, containing oligosaccharides smaller than maltotetraose, 8% $Ca^{2+}$-form resins are appropriate. For analysis of low-conversion syrups, 4% $Ag^{+}$-form resins are used. If samples are carefully de-ionized prior to injection, columns of this type can operate for years without significant loss of resolution, and are therefore often the choice for industrial, quality-control applications.

Much of the previous discussion is directly applicable to the methods that are available for the separation of other neutral oligosaccharides. Hence, those oligosaccharides produced from the partial hydrolysis of cellulose, xylans, and dextrans with acid have been separated on reversed-phase packings,[60,64,69,142] aminopropyl-bonded silica gel,[34,146–149] and cation-exchange resins.[13,49,150,151] Because of the low solubility of these oligosaccharides in partly aqueous, mobile phases, those stationary phases that use pure water as the mobile phase (cation exchangers and reversed-phase packings) are of especially great utility. The 4%-crosslinked, $Ag^{+}$- and $Ca^{2+}$-form resins are particularly well suited to separation of cello-oligosaccharides (d.p. 1–8 in <20 min) and can be regenerated or repacked by the user (see Section II,2,b). With proper care, aminopropyl silica-gel columns can also produce highly efficient and rapid (<15 min) separations of cello-oligosaccharides of d.p. 1–6, with the usual limitations imposed by low solubility of the cello-oligosaccharides in the mobile phase. Aminopropyl-bonded and physically modified amino silica-gel columns have also been used to separate fructo-oligosaccharides[137,152,153] from inulin (from d.p. 1 to 30 in <40 min), and oligosac-

(146) E. K. Gum, Jr., and R. D. Brown, Jr., *Anal. Biochem.*, 82 (1977) 372–375.
(147) R. Niesner, W. Brüller, and O. Bobleter, *Chromatographia*, 11 (1978) 400–402.
(148) D. Noel, T. Hanai, and M. D'Amboise, *J. Liq. Chromatogr.*, 2 (1979) 1325-1336.
(149) K. L. Smiley, M. E. Slodki, J. A. Boundy, and R. D. Plattner, *Carbohydr. Res.*, 108 (1982) 279–283.
(150) M. R. Ladisch, A. W. Anderson, and G. T. Tsao, *J. Liq. Chromatogr.*, 2 (1979) 745–760.
(151) J. Schmidt, M. John, and C. Wandrey, *J. Chromatogr.*, 213 (1981) 151–155.
(152) A. Heyraud, M. Rinaudo, and F. R. Taravel, *Carbohydr. Res.*, 128 (1984) 311–320.
(153) W. Praznik, R. H. F. Beck, and E. Nitsch, *J. Chromatogr.*, 303 (1984) 417–421.

charides produced from the hydrolysis of chitin.[154,155] The latter oligosaccharides have also been separated on reversed-phase columns.[59]

**d. Simple, Ionic Oligosaccharides.**—Several methods have been developed for the l.c. separation of simple, linear, acidic oligosaccharides that contain one or two types of carbohydrate unit. Oligosaccharides in this category include those produced by the partial depolymerization of pectic or alginic acids, κ-carrageenan, and similar polysaccharides. Traditionally, these oligomers have been separated by gel-filtration[156] or anion-exchange column-chromatography (see Ref. 156 and those cited therein). Both methods are lengthy, and, because of the tendency of oligoglycuronans to aggregate,[157] the former method may not give pure fractions. L.c. methods for the separation of normal, pectic oligoglycosiduronic acids and those possessing 4,5-unsaturated, terminal residues, employ weak and strong anion-exchange silica-,[67,158] strong anion-exchange resin-,[159] and reversed-phase silica-[67,160] based stationary phases. Strong anion-exchange silica gels, which provide excellent resolution of oligoglycuronans from d.p. 1 through 8 in <30 min (acetate buffers as the mobile phase) are relatively unstable, and have limited lifetimes. Weak anion-exchange silica gel (aminopropyl silica gel) eluted with acetate buffers provides somewhat poorer separations,[67] but is more stable. Strong-base anion-exchange resins (polystyrene-based), which are relatively stable and provide high-resolution separations,[159] have not been extensively applied to high-performance l.c. methods, but show considerable promise for future developments. Reversed-phase ($C_{18}$) silica gels are excellent stationary-phases for oligoglycuronan separations when a hydrophobic, ion-pairing reagent is used to suppress ionization of the carboxyl groups. Heyraud and Rochas[68] separated pectic oligosaccharides having d.p. 1–11 by using the ion-pairing reagent tributyldodecylammonium chloride. Voragen and coworkers[67] used tetrabutylammonium bromide to separate oligoglycosiduronic acids of d.p. 1–7 in <15 min. Although the $C_{18}$ columns are very stable under these conditions, it has been noted that the bromide salt of this reagent has a deleterious effect on chromatographic

(154) P. van Eikeren and H. McLaughlin, *Anal. Biochem.*, 77 (1977) 513–522.
(155) S. J. Mellis and J. U. Baenziger, *Anal. Biochem.*, 114 (1981) 276–280.
(156) J.-F. Thibault, *J. Chromatogr.*, 194 (1980) 315–322.
(157) M. A. F. Davis, M. J. Gidley, E. R. Morris, D. A. Powell, and D. A. Rees, *Int. J. Biol. Macromol.*, 2 (1980) 330–332.
(158) E. A. Nothnagel, M. McNeil, P. Albersheim, and A. Dell, *Plant Physiol.*, 71 (1983) 916–926.
(159) P. Vrátný, O. Mikeš, P. Štrop, J. Čoupek, L. Rexová-Benková, and D. Chadimová, *J. Chromatogr.*, 257 (1983) 23–35.
(160) T. Romeo and J. F. Preston III, *Carbohydr. Res.*, 153 (1986) 181–193.

hardware; use of the hydroxide is therefore recommended.[161] Other simple, acidic oligosaccharides derived from alginate[160] and κ-carrageenan[68] have also been separated by ion-pairing, reversed-phase chromatography.

Few reports exist on the l.c. separation of such cationic carbohydrate oligomers as those resulting from partial hydrolysis of chitosan. The neutral, *N*-acetylated analogs from chitin hydrolyses are, however, well separated on columns of aminopropyl silica gel[154,155] and reversed-phase silica gel.[59]

**e. Complex (and Cyclic), Neutral Oligosaccharides.**—This Section covers those non-ionic oligosaccharides (≥d.p.3) from various sources that are heterogeneous with respect to linkage or composition, or both. For a general overview of the use of various l.c. stationary phases in oligosaccharide separations, Section III,1,c should be referred to.

Such plant-derived oligosaccharides as those produced from the hydrolysis of heteropolysaccharides and various cell-wall carbohydrate polymers often contain several different sugars and linkages. For the separation of the simplest members of this class, namely, the trisaccharides, stationary phases of amine-modified silica gel[39,162] possess greater selectivity than cation-exchange resins, and are therefore recommended. In this system, retention of various trisaccharides is governed by sugar composition and linkage, and some of the precise effects of these parameters on retention have been determined.[88,162] For example, isomaltotriose and panose, which differ by only one linkage substitution, are readily separated on aminopropyl silica. Reversed-phase stationary phases ($C_{18}$ type) are useful for complex, plant oligosaccharides that are larger than tri- or tetra-saccharides. Retention on these columns is especially sensitive to saccharide linkage, and linear oligosaccharides are often well separated from those that are isomeric, but branched. Chromatography on reversed-phase packings was used to study the branching patterns of dextrans,[163] and to compare the structure of a synthetic *versus* a mycelial wall-derived hexa-*O*-β-D-glucopyranosyl-D-glucitol.[164] The complete separation of eight isomeric hexa-*O*-β-D-glucopyranosyl-D-glucitols required a preliminary fractionation on a polar, aminocyanopropyl silica-gel column, followed by a second separation on $C_{18}$-bonded silica

(161) A. G. J. Voragen, personal communication.
(162) Ž. L. Nikolov, M. M. Meagher, and P. J. Reilly, *J. Chromatogr.*, 321 (1985) 393–399.
(163) C. Taylor, N. W. H. Cheetham, and G. J. Walker, *Carbohydr. Res.*, 137 (1985) 1–12.
(164) J. K. Sharp, P. Albersheim, P. Ossowski, Å. Pilotti, P. Garegg, and B. Lindberg, *J. Biol. Chem.*, 259 (1984) 11,341–11,345.

gel.[165] Combined approaches of this type are often necessary for the complete separation of such complex mixtures. An alternative approach for the separation of plant and microbial oligosaccharides, pioneered by Albersheim and coworkers,[63,166] is to prepare the peralkylated derivatives of the oligosaccharides, which are subsequently fractionated on $C_{18}$ columns. Although extra time is required for the derivatization step, the resulting separated products may be directly analyzed by mass spectrometry, which can lead to determination of complete oligosaccharide sequence.

Numerous glycoprotein-derived oligosaccharides have been separated by l.c. techniques, and these methods may be divided into two general categories: those employing amine-modified silica gel and those with $C_{18}$ (reversed-phase) stationary phases. With these columns, various "high-mannose," "complex," "hybrid," and miscellaneous oligosaccharides from ovalbumin,[155,167] IgD,[168,169] IgM,[167] α-acid glycoprotein,[155,170] mucins,[171-174] ovarian-cyst glycoproteins,[174] ceruloplasmin,[155] β-D-glucosiduronase,[175] fibronectin,[176] slime-mold glycoprotein,[177] tumor cells,[178]

(165) J. K. Sharp, B. Valent, and P. Albersheim, *J. Biol. Chem.*, 259 (1984) 11,312–11,320.
(166) B. S. Valent, A. G. Darvill, M. McNeil, B. K. Robertsen, and P. Albersheim, *Carbohydr. Res.*, 79 (1980) 165–192.
(167) P. I. Clark, S. Narasimhan, J. M. Williams, and J. R. Clamp, *Carbohydr. Res.*, 118 (1983) 147–155.
(168) S. J. Mellis and J. U. Baenziger, *J. Biol. Chem.*, 258 (1983) 11,546–11,556.
(169) S. J. Mellis and J. U. Baenziger, *J. Biol. Chem.*, 258 (1983) 11,557–11,563.
(170) M. L. E. Bergh, P. L. Koppen, D. H. van den Eijnden, I. Arnarp, and J. Lönngren, *Carbohydr. Res.*, 117 (1983) 275–278.
(171) A. Boersma, G. Lamblin, P. Degand, and P. Roussel, *Carbohydr. Res.*, 94 (1981) C7–C9.
(172) H. Van Halbeek, L. Dorland, J. F. G. Vliegenthart, W. E. Hull, G. Lamblin, M. Lhermitte, A. Boersma, and P. Roussel, *Eur. J. Biochem.*, 127 (1982) 7–20.
(173) G. Lamblin, A. Boersma, M. Lhermitte, P. Roussel, J. H. G. M. Mutsaers, H. Van Halbeek, and J. F. G. Vliegenthart, *Eur. J. Biochem.*, 143 (1984) 227–236.
(174) V. K. Dua, V. E. Dube, and C. A. Bush, *Biochim. Biophys. Acta*, 802 (1984) 29–40.
(175) D. R. Howard, M. Natowicz, and J. U. Baenziger, *J. Biol. Chem.*, 257 (1982) 10,861–10,868.
(176) P. W. Ledger, S. K. Nishimoto, S. Hayashi, and M. L. Tanzer, *J. Biol. Chem.* 258 (1983) 547–554.
(177) C. A. Gabel, C. E. Costello, V. N. Reinhold, L. Kurz, and S. Kornfeld, *J. Biol. Chem.*, 259 (1984) 13,762–13,769.
(178) S. R. Hull, R. A. Laine, T. Kaizu, I. Rodriguez, and K. L. Carraway, *J. Biol. Chem.*, 259 (1984) 4866–4877.

ovomucoid,[179,180] blood-group glycoproteins,[181] and histocompatability antigens[182] have been separated, and often isolated (see also Section III,3.). Separations of these oligosaccharides on amine-modified silica gel with acetonitrile–water mobile phases were pioneered in 1981 by three separate groups,[155,171,183] and most of the reports of the separation of glycoprotein-derived oligosaccharides since published have been extensions of those methods. Oligosaccharides or oligosaccharide-alditols (produced by chemical cleavage of the oligosaccharide from glycoprotein under reductive conditions) having ~3 to 15 carbohydrate residues may usually be separated in <60 min on these columns by normal phase-partitioning mechanisms. Several factors[88,155,170,171,183] that govern the retention of these oligosaccharides are molecular weight, carbohydrate composition, anomeric linkage, and branching patterns. An important advantage of this system over the reversed-phase or cation-exchange type is the ability to modify and optimize the mobile-phase composition. A preliminary molecular weight (d.p.) fractionation of glycans, using a weak mobile phase (relatively high percentage of water) can be followed by a second fractionation of each collected peak on the same column, using a strong mobile phase (relatively high percentage of acetonitrile) to resolve isomers in each size-class. As previously stated, aminopropyl-bonded silica-gel columns are somewhat unstable and should always be used with the precautions outlined in Sections II,1,b and II,2,a. Turco[183] found that silica-gel columns modified *in situ* with 1,4-diaminobutane are more durable than covalently bonded amino columns for the separation of "high-mannose" and related oligosaccharides.

There have been relatively few examples of the use of reversed-phase columns[59,174,182] for separations of complex, glycoprotein-derived glycans. Retention in this system is based upon hydrophobic interactions between carbohydrate and stationary phase, and appears to be sensitive to oligosaccharide linkage, composition, and branching, rather than to molecular weight or number of sugar residues. Because most oligosaccharides are relatively polar, little interaction takes place between them and the sta-

(179) J. P. Parente, G. Strecker, Y. Leroy, J. Montreuil, and B. Fournet, *J. Chromatogr.*, 249 (1982) 199–204.

(180) J. P. Parente, J.-M. Wieruszeski, G. Strecker, J. Montreuil, B. Fournet, H. van Halbeek, L. Dorland, and J. F. G. Vliegenthart, *J. Biol. Chem.*, 257 (1982) 13,173–13,176.

(181) A. M. Wu, E. A. Kabat, B. Nilsson, D. A. Zopf, F. G. Gruezo, and J. Liao, *J. Biol. Chem.*, 259 (1984) 7178–7186.

(182) S. J. Swiedler, G. W. Hart, A. L. Tarentino, T. H. Plummer, Jr., and J. H. Freed, *J. Biol. Chem.*, 258 (1983) 11,515–11,523.

(183) S. J. Turco, *Anal. Biochem.*, 118 (1981) 278–283.

tionary phase, even when the weakest mobile-phase (water) is used. Reversed-phase columns are, therefore, most useful for separating those complex oligosaccharides that contain higher proportions of (nonpolar) acetyl and methyl groups, or deoxy sugar moieties, and those that have been derivatized with nonpolar groups to enhance detectability.

Other complex, neutral oligosaccharides that have been fractionated by l.c. techniques include those found in human milk[21,184,185] and in urine from patients with lysosomal-storage disorders.[6,186]

An unusual class of oligosaccharides that contain a homogenous sugar composition are the cyclic oligosaccharides, such as the cyclomalto-oligosaccharides[186a] (the "cyclodextrins"), having $\alpha$-(1 → 4)-linked D-glucosyl residues, and the cyclosophoroses, containing $\beta$-(1 → 2)-linked D-glucosyl residues. The most common cyclodextrins, those with 6, 7, or 8 D-glucosyl units, have been separated on amine-modified silica-gel columns,[187] calcium-form cation-exchange columns,[23,188] and reversed-phase silica-gel columns.[189] The cyclosophoroses, having a d.p. up to 40, have been separated[189–191] on amine-modified and reversed-phase silica-gel columns. All three stationary phases mentioned here separate the cyclic oligosaccharides by different mechanisms, and they provide a unique retention order. Separations that are sensitive to molecular weight are produced on amine-modified silica-gel phases, with large oligosaccharides being eluted last. With reversed phases, separations based on hydrophobicity occur, with the most hydrophobic, least water-soluble[189] compounds being eluted last. On cation-exchange resin columns, separations are based on a combination of size-exclusion and hydrophobic interactions. Koizumi and coworkers[189–191a] used the unique retention-mechanisms of these phases to obtain structural information about newly discovered cyclic oligosaccharides. For instance, the molecular weight

(184) H. Egge, A. Dell, and H. Von Nicolai, *Arch. Biochem. Biophys.*, 224 (1983) 235–253.
(185) V. K. Dua, K. Goso, V. E. Dube, and C. A. Bush, *J. Chromatogr.*, 328 (1985) 259–269.
(186) N. M. K. Ng Ying Kin and L. S. Wolfe, *Anal. Biochem.*, 102 (1980) 213–219.
(186a) See R. J. Clarke, J. H. Coates, and S. F. Lincoln, This Volume, pp. 205–249.
(187) H. Bender, *Carbohydr. Res.*, 124 (1983) 225–233.
(188) H. Hokse, *J. Chromatogr.*, 189 (1980) 98–100.
(189) K. Koizumi, Y. Okada, S. Horiyama, T. Utamura, M. Hisamatsu, and A. Amemura, *J. Chromatogr.*, 265 (1983) 89–96.
(190) M. Hisamatsu, A. Amemura, K. Koizumi, T. Utamura, and Y. Okada, *Carbohydr. Res.*, 121 (1983) 31–40.
(191) K. Koizumi, Y. Okada, T. Utamura, M. Hisamatsu, and A. Amemura, *J. Chromatogr.*, 299 (1984) 215–224.
(191a) K. Koizumi, T. Utamura, M. Sato, and Y. Yagi, *Carbohydr. Res.*, 153 (1986) 55–67.

(or d.p.), sugar composition, and type of linkages present in a cyclic oligosaccharide may be determined by comparing the l.c. analysis of its partial hydrolyzate to that of known, standard oligosaccharides. In addition, the same methods can provide information about branched structures. If branching is present in the cyclic oligosaccharide, l.c. analysis (amine-modified silica-gel phase) of the partial hydrolyzate will reveal multiple peaks of materials having the same approximate d.p. value. Analysis[191a] of these peaks can verify the presence of simple D-glucosyl or maltosyl side-chains. To identify more-complex branching patterns, additional information from methylation analysis or n.m.r. spectroscopy is required.

**f. Complex, Ionic Oligosaccharides and Glycopeptides.**—This Section will be divided into four separate areas: sialylated (or phosphorylated) oligosaccharides, glycopeptides, and acidic glycosaminoglycan and acidic plant cell-wall oligosaccharides.

The sialylated oligosaccharides of glycoproteins are of considerable biological interest, and this is reflected in the development of many l.c. methods for their fractionation. Free sialylated oligosaccharides from such biological sources as human milk,[192] the urine from patients with lysosomal-storage disease,[186,193] or *in vitro* sialyltransferase assays, are often encountered.[194,195] In other instances, the N- or O-linked oligosaccharides of glycoproteins are, before l.c. analysis, liberated by hydrazinolysis (and subsequent reduction),[193] or alkaline borohydride treatment,[196] respectively. In each case, the product is an acidic oligosaccharide-alditol, and it is this species that is most commonly chromatographed.

Three types of l.c. stationary phase have been used for sialylated oligosaccharide separation: weak anion-exchange silica gel (primary amine groups), strong anion-exchange silica-gel (quaternary amine groups), and strong anion-exchange resin beads. Such strong anion-exchangers as MicroPak-AX-10 are eluted with phosphate buffer gradients (pH 4) and they separate sialylated oligosaccharides according to the number of sialic acid

(192) J.-M. Wieruszeski, A. Chekkor, S. Bouquelet, J. Montreuil, J. Peter-Katalinic, and H. Egge, *Carbohydr. Res.*, 137 (1985) 127–138.
(193) S. J. Mellis and J. U. Baenziger, *Anal. Biochem.*, 134 (1983) 442–449.
(194) M. L. E. Bergh, P. L. Koppen, and D. H. van den Eijnden, *Biochem. J.*, 201 (1982) 411–415.
(195) M. L. E. Bergh, G. J. M. Hooghwinkel, and D. H. van den Eijnden, *J. Biol. Chem.*, 258 (1983) 7430–7436.
(196) D. K. Podolsky, *J. Biol. Chem.*, 260 (1985) 8262–8271.

groups bound.[197,198,198a] Such class-separated oligosaccharides are, however, generally heterogeneous with respect to carbohydrate composition and further chromotography on primary amine-bonded silica is necessary for obtaining pure, homogeneous fractions.[198,198a] In the latter case, the primary amine-bonded silica gel is usually eluted with acetonitrile mixed with one of the following buffers: ammonium hydrogencarbonate,[199] triethylammonium acetate,[193,196] sodium acetate,[186] potassium dihydrogenphosphate containing 0.01% of 1,4-diaminobutane,[198] or potassium phosphate. Under these conditions (pH 5.2–7.0), the acidic oligosaccharides are separated on the basis of carbohydrate content, linkage, and composition, allowing the separation of linkage isomers as well as of oligosaccharides of differing mass. A slight modification[199a] of these procedures has been used to differentiate between complex sialylated and sulfated oligosaccharides. Fractionation of acidic oligosaccharides may also be accomplished on polystyrene-based anion-exchangers eluted with sodium chloride gradients.[200,201] In this case, fractionation is similar to that on primary amine-bonded silica gel. In all of the foregoing instances, because of the extreme heterogeneity of many glycoprotein-derived oligosaccharides, a combination of two or three of the methods described, in conjunction with classical gel-filtration chromatography, is usually necessary for complete purification. Such coupled procedures have allowed the fractionation of sialylated oligosaccharides from bronchial,[172,199] submaxillary,[201,202] and colonic mucins,[196] tumor-cell glycoproteins,[203] fetuin,[195,197] ovomucoid,[198] orosomucoid,[197,198a] ovalbumin,[197] ceruloplasim,[193,197] fibronectin,[176] IgD (human),[168,169] glycocalicin,[200] and thyroglobulin.[201] In addition, phosphorylated oligosaccharides have been similarly fractionated.[193,197]

One of the most difficult aspects of the study of glycoprotein structure is the remarkable heterogeneity displayed in their glycan chains. The l.c.

(197) J. U. Baenziger and M. Natowicz, *Anal. Biochem.*, 112 (1981) 357–361.
(198) J. P. Parente, Y. Leroy, J. Montreuil, and B. Fournet, *J. Chromatogr.*, 288 (1984) 147–155.
(198a) P. Cardon, J. Paz Parente, Y. Leroy, J. Montreuil, and B. Fournet, *J. Chromatogr.*, 356 (1986) 135–146.
(199) G. Lamblin, A. Klein, A. Boersma, Nasio-ud-Din, and P. Roussel, *Carbohydr. Res.*, 118 (1983) c1–c4.
(199a) E. D. Green and J. U. Baenziger, *Anal. Biochem.*, 158 (1986) 42–49.
(200) T. Tsuji, S. Tsunehisa, Y. Watanabe, K. Yamamoto, H. Tohyama, and T. Osawa, *J. Biol. Chem.*, 258 (1983) 6335–6339.
(201) T. Tsuji, K. Yamamoto, Y. Konami, T. Irimura, and T. Osawa, *Carbohydr. Res.*, 109 (1982) 259–269.
(202) M. L. E. Bergh, P. Koppen, and D. H. van den Eijnden, *Carbohydr. Res.*, 94 (1981) 225–229.
(203) A. P. Sherblom and C. E. Dahlin, *J. Biol. Chem.*, 260 (1985) 1484–1492.

separation and isolation of hydrazine- or alkali-generated oligosaccharide-alditols from glycoproteins, as just described, is a useful technique, but it does not answer the question of whether all the oligosaccharides come from one, or from several, glycosylation site(s). Hence, as described next, several methods have been developed for the isolation of intact glycopeptides, released from the parent molecule by gentle, protease treatment. By this procedure, the entire glycosylation site(s), is separated or isolated (see Section III,3), allowing subsequent structural analysis of both the peptide and the carbohydrate portion.

Glycopeptides are usually separated on either reversed-phase or aminopropyl-bonded silica gel. The former stationary phase is usually eluted with an organic modifier, such as acetonitrile, in conjunction with an aqueous buffer at pH 7–7.5 (phosphate[204,205] or borate[206,207] type) or pH 2–3 (phosphate[208,209], phosphate–chlorate,[16,182] or trifluoroacetate type[210]). Under these conditions, glycopeptides are separated largely on the basis of peptide variation. This is supported by the fact that two, nearly identical, 13-amino acid peptides having greatly differing glycan chains (monosaccharide *versus* pentasaccharide chain) are only marginally separated by this system,[205] whereas large glycopeptides that differ in only a single amino acid residue are separated with relative ease.[210] These characteristics make these systems ideal for the separation of glycosylation sites, that is, homogeneous-peptide regions that contain one homogeneous or heterogeneous glycan chain. Once the homogeneous-peptide sequences (glycosylation sites) have been separated, or isolated, the heterogeneity of the glycan chains may be studied by classical, size-exclusion chromatography after peptide hydrolysis,[208] or by l.c. analysis. With the latter method, several procedures may be used, including reversed-phase chromatography under special conditions. Because of the low sensitivity of reversed-phase columns to carbohydrate variation, it is necessary to use combinations of weak solvents and elution times[207] of up to 8 h. In special cases where all of the peptide except for one amino acid may be enzymically digested away, as in ovalbumin,[62] the carbohydrate heterogeneity may be moderately resolved by using reversed-phase columns eluted with phosphate buffer alone.

(204) T. Takahashi, P. G. Schmidt, and J. Tang, *J. Biol. Chem.*, 258 (1983) 2819–2830.
(205) T. Takahashi, P. G. Schmidt, and J. Tang, *J. Biol. Chem.*, 259 (1984) 6059–6062.
(206) H. Iwase, S.-C. Li, and Y.-T. Li, *J. Chromatogr.*, 267 (1983) 238–241.
(207) H. Iwase, Y. Kato, and K. Hotta, *J. Chromatogr.*, 320 (1985) 426–429.
(208) P. Hsieh, M. R. Rosner, and P. W. Robbins, *J. Biol. Chem.*, 258 (1983) 2548–2554.
(209) P. Hsieh, M. R. Rosner, and P. W. Robbins, *J. Biol. Chem.*, 258 (1983) 2555–2561.
(210) D. Tetaert, N. Takahashi, and F. W. Putnam, *Anal. Biochem.*, 123 (1982) 430–437.

As in most of the other complex separations described herein, a combined separation approach is usually needed in order to ensure homogeneity in glycopeptide fractions. Hence, after separation of glycosylation-site glycopeptides by one of the reversed-phase systems previously described, the oligosaccharides may be released by chemical or enzymic treatment, and then separated by one of the methods presented in Section III,1,e. Alternatively, these semipurified glycoprotein fractions may be resolved on primary amine-type silica-gel columns, which separate glycopeptides according to variations in carbohydrate, rather than peptide, structure.[186,193] The methods just described have been used for the separation of glycopeptides from ovalbumin,[62,193,206,207] ribonuclease B,[193] cathepsin-D[204] and -B,[205] virus glycoproteins,[208] tumor-cell antigens,[182] ovomucoid,[211] orosomucoid,[206] ceruloplasmin,[210] immunoglobulins,[210] and urine of patients with pathological states.[186] Sialylated glycopeptides are separated by these systems, with minor modifications,[186,206] as are methylated[211] and *N*-acylated glycopeptides.[193]

Oligosaccharides produced by the enzymic or chemical hydrolysis of glycosaminoglycuronans have been separated by l.c. methods based on size-exclusion,[212,213] weak anion-exchange,[214] strong anion-exchange,[213,215,216] and normal phase-partition chromatography.[217] Although both sulfated and non-sulfated oligosaccharides from these biopolymers have been chromatographed, most methods have been developed for the latter type, as, for example, the hyaluronic acid-derived oligosaccharides.[213,214,217] Generally, these methods have been used to separate the odd- and even-numbered, oligosaccharides (composed of 2-acetamido-2-deoxy-D-glucopyranose and D-glucopyranuronic acid residues) produced by the action of various endo- and exo-glycanohydrolases on purified hyaluronic acid. Size-exclusion[212] methods allow the simultaneous quantification of mono-, di-, tetra-, to hexa-saccharides, and unreacted biopolymer. Odd- and even-numbered hyaluronate oligosaccharides (up to decasaccharide) may be separated with much higher resolution, however, on high-performance anion-exchange columns,[213,214] which separate oligosaccharides by a combination of molecular weight and charge effects. In addition, retention times of hyaluronate oligosac-

(211) J. Conchie, A. J. Hay, and J. A. Lomax, *Carbohydr. Res.*, 112 (1983) 281–295.
(212) P. J. Knudsen, P. B. Eriksen, M. Fenger, and K. Florentz, *J. Chromatogr.*, 187 (1980) 373–379.
(213) P. Nebinger, *J. Chromatogr.*, 320 (1985) 351–359.
(214) P. Nebinger, M. Koel, A. Franz, and E. Werries, *J. Chromatogr.*, 265 (1983) 19–25.
(215) S. R. Delaney, H. E. Conrad, and J. H. Glaser, *Anal. Biochem.*, 108 (1980) 25–34.
(216) M. J. Bienkowski and H. E. Conrad, *J. Biol. Chem.*, 260 (1985) 356–365.
(217) I. Takazono and Y. Tanaka, *J. Chromatogr.*, 288 (1984) 167–176.

charides in these chromatographic systems may be predicted by additive contributions of the constitutive groups.[218] A disadvantage of anion-exchange chromatographic separations of hyaluronate oligosaccharides (particularly on Dowex-1 X8 resin) is the extremely low recovery (<60%) of sample from the column effluent.

Chromatographic separations of sulfated glycosaminoglycuronan oligosaccharides are more difficult than those of the hyaluronate type, because of the complex variety of sulfation patterns that may exist. In the separation of oligosaccharides produced from nitrous acid-depolymerized heparin[216] on strong anion-exchange columns, for example, more than 16 different tetrasaccharides (differing in uronic acid composition and sulfation patterns) were resolved and isolated. The method provides high-resolution separations, but requires long run-times (>2 hr) and pre-chromatographic separation of oligosaccharides according to size (by gel-filtration). Nonetheless, it is a powerful tool for sequence analyses of heparin structure, and has also been applied to the separation of enzyme-generated heparin oligosaccharides.[219,220] Likewise, oligosaccharides from chondroitin sulfate show considerable heterogeneity in sulfation patterns, even in samples of "pure" chondroitin 4- or 6-sulfate. The variety of sulfation patterns in tetra- to deca-saccharides has been demonstrated by the separation of multiple "hybrid" oligosaccharides (of identical mass) by chromatography on strong anion-exchange silica-gel eluted with phosphate buffer.[215]

Complex, acidic oligosaccharides from plant cell-walls have occasionally been fractionated by l.c. methods. Because of the great structural heterogeneity of these oligosaccharides, l.c. is generally preceded by one of several additional fractionation steps, namely, gel filtration, ion-exchange, or affinity chromatography. Although there is one report of the fractionation of pectic-type oligosaccharides on strong anion-exchange silica-gel columns,[158] the majority of methods reported describe the fractionation of these molecules as their carboxyl-reduced, peralkylated derivatives on reversed-phase silica gel.[17,18,166] These methods have been used for fractionating oligosaccharides from the plant cell-wall polysaccharides rhamnogalacturonan I (Ref. 18) and II (Ref. 17), and have also been useful for the fractionation of various acidic fragments of bacterial, extracellular polysaccharides.[63]

(218) E. Shimada and G. Matsumara, *J. Chromatogr.*, 328 (1985) 73–80.
(219) K. G. Rice, Y. S. Kim, A. C. Grant, Z. M. Merchant, and R. J. Linhardt, *Anal. Biochem.*, 150 (1985) 325–331.
(220) Z. M. Merchant, Y. S. Kim, K. G. Rice, and R. J. Linhardt, *Biochem. J.*, 229 (1985) 369–377.

## 2. Additional, Selected Applications

**a. Analysis of Carbohydrates in Foods.**—The determination of simple and complex carbohydrates in foods by l.c. has been aptly discussed in reviews,[29,30,221,222] and will be covered here in only a general manner. In addition, reviews of this topic continue to appear on a biannual basis.[223–225] The carbohydrates in foods may be divided into two categories,[30] the (mostly) soluble, metabolically utilizable sugars and starches, and the insoluble, complex "dietary-fiber" carbohydrate that is resistant to human alimentary enzymes. It is the former class of carbohydrates that have received the most attention, and many l.c. methods for their determination exist (see Table II). Most of these applications require l.c. columns packed with either cation-exchange resins or amine-modified silica gel, which provide two different types of selectivities, often leading to complementary information. Because no single type of column will provide the selectivity and resolution required for every food analysis, some care must be taken in choosing the correct column for each application. In general, when an application can be served with a cation-exchange resin column, it should be the column of choice, because of the durability, ease of regeneration, rapid analyses, and complete recovery of carbohydrate from the column. In addition, these columns require pure water or dilute mineral acid as the mobile phase, which is economical and allows high-sensitivity detection on refractive index monitors. With proper, sample pre-treatment and the use of pre-columns (see Sections II,1,b and 2,b) cation exchange columns in the $H^+$, $Ag^+$, $Ca^{2+}$, or $Pb^{2+}$ form can be operated for several years without noticeable loss in efficiency. Saccharides are separated on these systems by a combination of size-exclusion and ligand-exchange mechanisms (see Section II,2,b) and columns with different metal-ion ligands give different selectivities that can be used to optimize specific separations. As summarized previously,[30] $Ag^+$ columns are optimized for the separation of oligosaccharides, such as

(221) W. A. Davis and C. G. Hartford, in G. Charalambous (Ed.), *Liquid Chromatographic Analysis of Food and Beverages*, Vol. 2, Academic Press, New York, 1979, pp. 353–362.
(222) R. Macrae, in G. Birch (Ed.), *Analysis of Food Carbohydrates*, Elsevier Appl. Sci., London, 1985, pp. 61–89.
(223) K. G. Sloman, A. K. Foltz, and J. A. Yeransian, *Anal. Chem.*, 53 (1981) 247R–248R, 265R–266R.
(224) A. K. Foltz, J. A. Yeransian, and K. G. Sloman, *Anal. Chem.*, 55 (1983) 169R–171R, 187R–188R.
(225) J. A. Yeransian, K. G. Sloman, and A. K. Foltz, *Anal. Chem.*, 57 (1985) 286R–288R, 306R–307R.

TABLE II
**Selected L. C. Methods for Soluble Food-Carbohydrates**

| Type of Food | Carbohydrates Analyzed |
|---|---|
| Beer, wort[151,226] | simple sugars and oligosaccharides |
| Cereal (breakfast)[227,228] | fructose, glucose, sucrose, maltose |
| Chewing gum[229] | sugar alcohols |
| Confectionery[230,231] | simple sugars |
| Corn syrup[50,145,232] | simple sugars, psicose, maltulose, oligosaccharides |
| Fruit juice[227,233,234] | sucrose, glucose, fructose |
| Honey,[227] invert sugar,[232,235] maple syrup,[227] molasses[236] | glucose, fructose |
| Ice cream,[227,237] lactose hydrolyzates,[57,238] milk,[38,239] whey,[85,238] lactulose syrups[240,241] | lactose, lactulose, galactose, glucose |

those in corn syrups. Columns in the $H^+$ form are especially useful for separation of simple sugars, sugar acids, and sugar alcohols that are present in fermented dairy products. The so-called "heavy-metal" or $Pb^{2+}$ forms of cation-exchange resins are useful for the separation of those monosaccharides found in dietary-fiber carbohydrates. The $Ca^{2+}$-form columns are useful for the class separation of mono-, di-, tri-, and tetrasaccharides and sugar alcohols.

(226) G. K. Buckee and D. E. Long, *J. Am. Soc. Brew. Chem.*, 40 (1982) 137–140.
(227) J. L. Iverson and M. P. Bueno, *J. Assoc. Off. Anal. Chem.*, 64 (1981) 139–143.
(228) L. C. Zygmunt, *J. Assoc. Off. Anal. Chem.*, 65 (1982) 256–264.
(229) E. C. Samarco and E. S. Parente, *J. Assoc. Off. Anal. Chem.*, 65 (1982) 76–78.
(230) S. I. M. Johncock and P. J. Wagstaffe, *Analyst*, 105 (1980) 581–588.
(231) D. J. Timbie and P. G. Keeney, *J. Food Sci.*, 42 (1977) 1598–1599.
(232) H. D. Scobell, K. M. Brobst, and E. M. Steele, *Cereal Chem.*, 54 (1977) 905–917.
(233) J. K. Palmer and W. B. Brandes, *J. Agric. Food Chem.*, 22 (1974) 709–712.
(234) P. E. Shaw and C. W. Wilson, III, *J. Sci. Food Agric.*, 34 (1983) 109–112.
(235) M. Wnukowski, *Int. Sugar J.*, 86 (1984) 170–174.
(236) C. E. Damon and B. C. Pettitt, Jr., *J. Assoc. Off. Anal. Chem.*, 63 (1980) 476–480.
(237) J. M. Beebe and R. K. Gilpin, *Anal. Chim. Acta*, 146 (1983) 255–259.
(238) I. J. Jeon, S. J. Galitzer, and K. J. Hennessy, *J. Dairy Sci.*, 67 (1984) 884–887.
(239) J. F. Pirisino, *J. Food Sci.*, 48 (1983) 742–744, 754.
(240) L. A. T. Verhaar, M. J. M. Van Der Aalst, J. A. W. M. Beenackers, and B. F. M. Kuster, *J. Chromatogr.*, 170 (1979) 363–370.
(241) F. W. Parrish, K. Hicks, and L. Doner, *J. Dairy Sci.*, 63 (1980) 1809–1814.

On $Ca^{2+}$-form columns, some separation of monosaccharides is possible and, for the separation of galactose and glucose in dairy products, this is the column of choice. The separation of several disaccharides, such as sucrose plus maltose plus lactose, in sweetened dairy products cannot be accomplished on single-resin columns, however, and separation on amine-modified silica gel or on dual-resin columns[241a] is recommended. These columns are capable of separating the five major food sugars, namely, D-glucose, D-fructose, sucrose, maltose, and lactose, but are subject to rapid degradation if proper precautions are not used (see Section II,2,a).

Amine modifiers in the mobile phase have been used in conjunction with silica-gel columns[31] to provide more-stable columns. In addition, the use of diol-type silica-gel columns for the separation of food sugars has been reported.[38] These columns provide separations similar to those afforded by aminopropyl silica gel, but they are much more stable. The use of these diol columns, whenever practical, is therefore recommended.

**b. Analysis of Carbohydrates in Biomass-Conversion Processes.**—In biomass "conversion," plant-derived polysaccharides are partially or totally converted into fermentable sugars by the aid of chemical or enzymic methods. L.c. methods are useful in monitoring such reactions, because they can provide several different types of information, namely, the extent of polysaccharide hydrolysis, the distribution of the various monosaccharides produced, and the amounts of sugar fermentation- or degradation-products, or both, that are present.

For monitoring the extent of polysaccharide hydrolysis, l.c. methods that separate and analyze the non-fermentable oligosaccharides (d.p. 3–30) derived from cellulose, hemicellulose, and pectins are useful, and have already been described (see Section III,1,c). For determination of the monosaccharide composition of completely hydrolyzed, plant polysaccharides, l.c. is especially useful and has been applied to the compositional analysis of hydrolyzed plant fiber,[242] wood pulps,[83,243] plant cell-walls,[244] and cotton fibers.[245] In these representative examples, the major sugars of interest, namely, glucose, xylose, galactose, arabinose, and mannose, have traditionally been difficult to resolve by l.c. The separa-

(241a) J. A. M. van Riel and C. Olieman, *J. Chromatogr.*, 362 (1986) 235–242.
(242) J. L. Slavin and J. A. Marlett, *J. Agric. Food Chem.*, 31 (1983) 467–471.
(243) F. E. Wentz, A. D. Marcy, and M. J. Gray, *J. Chromatogr. Sci.*, 20 (1982) 349–352.
(244) W. Blaschek, *J. Chromatogr.*, 256 (1983) 157–163.
(245) W. Schwald, R. Concin, G. Bonn, and O. Bobleter, *Chromatographia*, 20 (1985) 35–40.

tion of these sugars on high-performance aminopropyl silica-gel columns is dependent on the source and condition of the column. Although Binder[33] was able to separate these sugars efficiently, Yang and coworkers[246] had to couple two such columns together in order to achieve satisfactory resolution. Although separation was complete, the peaks were broad, and the detector sensitivity was poor. Moreover, under these conditions, the pentoses and certain hexoses in those samples are known[10] to form covalent linkages with the amino groups of the stationary phase, which leads to improper quantitation and column failure. Separation of these sugars can be accomplished more efficiently on special cation-exchange resins ($Pb^{2+}$ form).[242,243] The accuracy of these methods has been shown to be the equal of that of official, paper-chromatographic methods, and they are much more convenient.[83] When samples contain rhamnose, in addition to the other five sugars already listed, total separation is not possible on $Pb^{2+}$-form columns,[242] and coupled aminopropyl silica-gel columns[246] or sequential separations[244] on aminopropyl silica gel, and $Pb^{2+}$-form cation-exchange columns are required.

Biomass samples that have been hydrolyzed by hydrothermal[13,247] or acid[19] treatment, and the products then fermented[13,22] contain a complex array of alcohols, acids, aldehydes, ketones, and various sugar degradation-products. For analysis of these mixtures, cation-exchange resin columns are often more useful than reversed-phase or amine-modified silica-gel types.[54] Various ionic forms of cation- exchange resin columns are used to determine sugar degradation-products, such as 2-furaldehyde and 5-(hydroxymethyl)-2-furaldehyde,[13,19,22,54,245,246] methylglyoxal,[245] so-called anhydroglucose,[245] and acetic and formic acids.[19] Using an $H^+$-form column, Patrick and Kracht[19] determined 2-furaldehyde, 5-(hydroxymethyl)-2-furaldehyde, total monosaccharides, and acetic acid in wood hydrolyzates in <20 min. By using the more-selective, $Pb^{2+}$-form of a similar resin, Schwald and coworkers[245] were able to separate the individual sugars and the furfurals in a plant hydrolyzate, but, on this column, the acids could not be determined. Proper column selection, therefore, depends on the information that must be obtained in each specific analysis.

Liquid-chromatographic techniques have been useful for studying the mode of action of various polysaccharide-degrading enzymes. Rapid and quantitative, l.c. end-product analysis has been used to study the action

(246) M. T. Yang, L. P. Milligan, and G. W. Mathison, *J. Chromatogr.*, 209 (1981) 316–322.

(247) G. Bonn and O. Bobleter, *Chromatographia*, 18 (1984) 445–448.

patterns of cellulases,[22,150,248] xylanases,[151] endo-glucanases, and poly(galacturonic acid) lyases[249] (see Addendum).

In addition to the normally suggested, sample pre-treatment procedures used prior to l.c. injection to avoid column contamination, $C_{18}$ bonded silica-gel cartridges[13] are particularly useful for removal of lignin-derived degradation-products that occur in biomass-conversion samples.

**c. Compositional Analysis of Carbohydrate Polymers.**—Determination of the monosaccharide composition of complex heteropolysaccharides, glycan chains, and glycoconjugates is of fundamental importance for studying the biological and physicochemical properties of these polymers. For this purpose, many excellent methods based upon gas–liquid chromatography (g.l.c.) currently exist. High-performance liquid-chromatographic methods should not be regarded as replacements for these methods, but as being complementary to them. The primary *raison d'être* for l.c. is to allow rapid, high-resolution separations of molecules without need for pre-derivatization. Accordingly, the most useful l.c. methods take this into consideration. In the compositional analysis of complex carbohydrate, both g.l.c. and l.c. methods have at least one thing in common; they must both be preceded by an enzymic or chemical hydrolysis step. In many cases, the limiting factor in the accuracy of compositional analysis is the method used to hydrolyze the specific polymer; this topic is outside the scope of this chapter, however, and only general guidelines will be given.

The monosaccharide composition of plant polysaccharides, for instance, is still usually accomplished by g.l.c. analysis, although progress is being made in l.c. separations. No method currently exists for the simultaneous determination of underivatized acidic and neutral sugars found in plant cell-wall polysaccharides. Sugar acids, such as the uronic acids produced by the hydrolysis of alginates, gums, and pectins, may be directly separated and quantitated by anion-exchange[67,71,96,250] and other[48] l.c. methods. The simultaneous separation of all neutral sugars present in plant cell-wall polysaccharides is also a particularly difficult challenge, and early attempts[246,251] led to only moderate separations between the various deoxy and neutral sugars present. A subsequent method,[244] utilizing both aminopropyl silica-gel and cation-exchange columns, now per-

(248) S. P. Shoemaker and R. D. Brown, Jr., *Biochim. Biophys. Acta*, 523 (1978) 133–146.
(249) A. R. White, A. G. Darvill, W. S. York, and P. Albersheim, *J. Chromatogr.*, 298 (1984) 525–530.
(250) G. Annison, N. W. H. Cheetham, and I. Couperwhite, *J. Chromatogr.*, 264 (1983) 137–143.
(251) E. C. Conrad and J. K. Palmer, *Food Technol. (Chicago)*, 30 (1976) 84, 86, 88–92.

mits the complete separation and quantitation of the major sugars (L-rhamnose, L-arabinose, D-xylose, D-mannose, D-galactose, and D-glucose) that exist in plant cell-wall hydrolyzates.

Because of their biomedical importance, several methods have been developed to determine the composition of various glycosaminoglycans, such as heparin, heparin sulfate, the chondroitin sulfates, dermatan sulfate, and hyaluronic acid. These polymers are composed basically of repeating disaccharide units, usually containing a hexuronic acid and a 2-amino-2-deoxyhexose, both of which may bear substituent groups (*N*- or *C*-sulfates and *N*-acetates). Fortunately, in most cases, these polymers can be uniformly degraded by specific chondroitinases, heparinases, heparanases, and hyaluronidases, or by specific chemical reagents, such as nitrous acid. Separation and identification of the reaction products (mostly disaccharides), by l.c. reveals the composition of the original polymer. Such analyses have been useful for determining the ratios of isomeric chondroitin or dermatan sulfates[126,127,252] and hyaluronic acid[212,217,253] in various tissues and physiological fluids. Moreover, the content of "over-sulfated" residues, a phenomenon related to pathological conditions,[130] may also be determined by the direct l.c. separation of the mono-, di-, and tri-sulfated disaccharides from various glycosaminoglycans.[128,130,131] The position of sulfate substitution on disaccharides may be determined by comparing l.c. retention-times before and after treatment with specific chondroitinsulfatases.[129,131] Because of the heterogeneity found in the carbohydrate composition, and the relatively high resolving power of l.c., as many as 9 different chondroitin disaccharides have been separated and identified.[131] Disaccharides produced from nitrous acid-[133,254] or enzyme-[132,220,255] degraded heparin and heparan sulfate have been separated by liquid-chromatographic procedures. Fluorescent derivatives of heparin disaccharides have also been prepared, to enhance their detectability[256] (see Addendum).

Through the combined approach of l.c. and the sequential applications of specific glycosaminoglycan-lyases, it is possible to determine the levels of several different glycosaminoglycans in the same tissue-sample. Thus, Gurr and coworkers[257] demonstrated that hyaluronate, chondroitin sulfate

(252) G. J.-L. Lee and H. Tieckelmann, *J. Chromatogr.*, 222 (1981) 23–31.
(253) A. Hjerpe, C. A. Antonopoulos, and B. Engfeldt, *J. Chromatogr.*, 245 (1982) 365–368.
(254) S. R. Delaney and H. E. Conrad, *Biochem. J.*, 209 (1983) 315–322.
(255) G. J.-L. Lee, D.-W. Liu, J. W. Pav, and H. Tieckelmann, *J. Chromatogr.*, 212 (1981) 65–73.
(256) M. Kosakai and Z. Yosizawa, *J. Biochem. (Tokyo)*, 92 (1982) 295–303.
(257) E. Gurr, G. Pallasch, S. Tunn, C. Tamm, and A. Delbrück, *J. Clin. Chem. Clin. Biochem.*, 23 (1985) 77–87.

isomers, and dermatan sulfate in human cartilage could be determined by sequential application of chondroitinase AC and chondroitinase ABC to the sample, followed each time by l.c. analysis of the enzyme products.

Because of their importance to medicine, biochemistry, and biotechnology, glycoproteins are of considerable interest, and accurate compositional analysis of the carbohydrates in these polymers is frequently needed. As with the other macromolecules previously discussed, there is a need for better methods for ensuring complete and quantitative hydrolysis. Because of the diversity in saccharide composition (neutral, acidic, and amino sugars), it is difficult to find optimal hydrolysis conditions. Some progress has been made in this area, and such reagents as anhydrous hydrogen fluoride[258,259] and others (see Refs. 81 and 260) may be capable of completely hydrolyzing glycosidic linkages without causing degradative reactions. Another, more frequently used, alternative to acid hydrolysis of glycoproteins is methanolysis.[92,261] This procedure may give improved yields of liberated monosaccharides, but it results in the formation of several (2 to 4) anomeric forms of glycoside of each, each of which gives rise to a separate l.c. peak, and this makes quantitation difficult.

Once a suitable hydrolysis procedure has been established, several useful l.c. procedures are available for saccharide separation, and the final choice in method should be based upon the sensitivity and selectivity required. Sensitivity of detection is a major problem, as many glycoprotein samples are available only in sub-milligram quantities. To improve detector-sensitivity, several precolumn derivatization methods have been developed (see Section IV,1e). Saccharides from glycoproteins have been converted into perbenzoates,[15,261,262] dansylhydrazones,[263] and other derivatives[264] which may be detected at the nanomole to picomole levels. Unfortunately, these added, bulky, chromophoric groups often overshadow the subtle stereochemical features of the original sugar, and separation (selectivity) of these derivatives is often poor. For this reason, l.c. methods that separate the underivatized carbohydrates and use post-column, detection-enhancement methods often give the best separations. One such method[80] claimed to separate, on a normal-phase column, all hexoses, amino sugars, and sialic acids known to be present in glycopro-

(258) A. J. Mort, *Carbohydr. Res.*, 122 (1983) 315–321.
(259) M. P. Sanger and D. T. A. Lamport, *Anal. Biochem.*, 128 (1983) 66–70.
(260) V. N. Reinhold, E. Coles, and S. A. Carr, *J. Carbohydr. Chem.*, 2 (1983) 1–18.
(261) N.. Jentoft, *Anal. Biochem.*, 148 (1985) 424–433.
(262) R. Oshima and J. Kumanotani, *J. Chromatogr.*, 265 (1983) 335–341.
(263) F. M. Eggert and M. Jones, *J. Chromatogr.*, 333 (1985) 123–131.
(264) G. Rosenfelder, M. Mörgelin, J.-Y. Chang, C.-A. Schönenberger, D. G. Braun, and H. Towbin, *Anal. Biochem.*, 147 (1985) 156–165.

teins. Another method[262] must be used in conjunction with this procedure to differentiate between free and *N*-acetylated amino sugars.

**d. Structural and Sequence Analysis of Simple and Complex Carbohydrates.**—L.c. methodology has played a key role in research on the structure of simple and complex carbohydrates and glycoconjugates. Besides providing high-resolution separation of these molecules for analytical purposes (see appropriate previous Sections), these nondestructive techniques serve as preparative methods (see Section III,3) for the efficient isolation of molecules that can subsequently be identified by chemical and instrumental means. In addition to these quantitative and preparative aspects, l.c. can provide qualitative information about the structure of various carbohydrates. A representative list of some of these methods is given in Table III. Most of the methods rely on additional enzymic procedures, or specific detection-systems, such as polarimetric[267] or mass-spec-

TABLE III
**L. C. Methods for Determining Structure or Sequence or Both in Carbohydrates**

| Determination | Type of Carbohydrate |
|---|---|
| Enantiomeric form | general review,[265] monosaccharides,[266] partially methylated monosaccharides[267] |
| Anomeric form | mono- and di-saccharides enzymically released from polysaccharides[267a] |
| Molecular weight | cyclodextrins,[189,190] linear oligosaccharides[268] |
| Glycosyl sequence in oligo- and poly-saccharides | general methods,[63,166] neutral oligosaccharides,[260,269,270] polysaccharide side-chains,[163,271] glycoprotein-derived oligosaccharides,[167,272] hyaluronic acid,[212,214,218] chondroitin sulfate,[215] heparin,[216,256] complex plant-polysaccharides,[17,18,63,166,271] complex bacterial-polysaccharides[63,166,273,274] |

(265) M. R. Little, *J. Biochem. Biophys. Methods*, 11 (1985) 195–202.
(266) J. Golik, H.-W. Liu, M. Dinovi, J. Furukawa, and K. Nakanishi, *Carbohydr. Res.*, 118 (1983) 135–146.
(267) A. Heyraud and P. Salemis, *Carbohydr. Res.*, 107 (1982) 123–129.
(267a) J. O. Baker and M. E. Himmel, *J. Chromatogr.*, 357 (1986) 161–181.
(268) S. Hase, T. Ikenaka, and Y. Matsushima, *J. Biochem. (Tokyo)*, 90 (1981) 1275–1279.
(269) S. F. Osman and P. D. Hoagland, *Carbohydr. Res.*, 128 (1984) 361–365.
(270) W. T. Wang, N. C. LeDonne, Jr., B. Ackerman, and C. C. Sweeley, *Anal. Biochem., 141 (1984) 366–381.*
(271) M. McNeil, A. G. Darvill, and P. Albersheim, *Plant Physiol.*, 70 (1982) 1586–1591.
(272) P. W. Tang and J. M. Williams, *Carbohydr. Res.* 136 (1985) 259–271.
(273) P. Åman, M. McNeil, L.-E. Franzén, A. G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 95 (1981) 263–282.
(274) W. Dudman, L.-E. Franzén, M. McNeil, A. G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 117 (1983) 169–183.

trometric detectors,[63] to provide structural information, but most of them would not be possible, or at least practical, without the use of modern l.c. equipment.

Especially noteworthy are the methods for determining the sequence of glycosyl residues in a complex oligo- or poly-saccharide. Methods of this type generate a series of overlapping oligomers that are separated and identified by l.c., in conjunction with either direct or indirect mass spectrometry. Most of these methods are still in the developmental stages, and they often require expensive and not-routinely available equipment (see also, Section IV,2) (see Addendum).

### 3. Preparative, Liquid Chromatography

Much of the research on the l.c. of carbohydrates has focused on analytical, rather than preparative, aspects. In reality, however, the conditions found in the majority of l.c. methods, namely, no sample derivatization, high-resolution separations, and nondestructive detection-techniques, are ideal for the preparation of pure molecules. Thus, most of the analytical l.c. methods previously described can also be used to isolate small quantities of pure compounds. This Section will cover the use of analytical-scale equipment for preparative applications, as well as the use of large-scale and "dedicated" preparative instruments for this purpose. Prior to discussion of these applications, a general overview of the preparative l.c. of carbohydrates will be given.

**a. General Aspects of Preparative L.C.**—The area of preparative l.c. has been covered in two reviews.[24,275] Investigators have developed theories to predict resolution of components at various $\kappa'$ values, loading capacities, flow rates, theoretical plates, sample volumes, and several other column parameters. The effects of these parameters on preparative, chromatographic resolution is complex and thus far unpredictable, and, as a result, preparative l.c. remains a highly empirical process. Optimal preparative l.c. is achieved when the largest amount of *pure* sample can be obtained in the *shortest* time. These optimum conditions are reached only by repeated experimentation and parameter-modification. Because the goal of preparative l.c. is the preparation of pure compounds, a small, high-resolution system will often be more efficient than a large, low-resolution chromatograph which produces fractions that must be repurified.

(275) M. Verzele and C. Dewaele, *LC Mag.*, 3 (1985) 22–28.

All of the stationary phases previously mentioned may be used for preparative purposes. Aminopropyl-bonded silica gels have the greatest sample loadability[39] of any of the phases, but, often, the carbohydrates to be isolated have low solubility in the acetonitrile–water mobile phases used in conjunction with them. When using these columns in the preparative mode, care must be taken to use conditions[10] that prevent glycosylamine formation between stationary-phase amino groups and reducing sugars. Reactive sugars, such as galactose and ribose, should not be isolated on these columns. Amine modifiers should not be used in mobile phases for preparative chromatography of reducing carbohydrates.

Cation-exchange resin and $C_{18}$-bonded silica columns have lower sample capacities[39,64,143] than amine-modified silica-gel columns, but they are more robust, do not covalently interact with sample components, and can be eluted with pure water at low flow-rates. Water is an ideal mobile-phase, because of low cost, solute solubility, and ability to be completely removed from collected samples by evaporation. All mobile phases should have these characteristics—including buffers, which should contain volatile components.[25,199]

The level of sample recovery from columns should always be noted. Low recovery may lead to rapid column-failure. Low recovery-values (50–90%) have often been reported[61,165] for normal- and reversed-phase columns. Preparative samples should always be pretreated by passage through a column (by gravity or suction) of a type of bonded phase similar to that used in the preparative separation. This packing material can generally be prepared, or purchased, at nominal cost. Pre-columns are generally recommended for preparative chromatography, but they are often less effective than the pretreatment just described, and they may be degraded quickly.

Occasionally, silica gel and bonded-phase material may be leached from the column and be introduced into the purified sample.[6,174] This may be removed by centrifugation, filtration, or extraction of the sample with nonpolar solvents, such as hexane.

**b. Preparative L.C. in Analytical-Scale Equipment.**—The majority of preparative l.c. is carried out on this level. No changes in hardware (except a larger sample-injector loop), column, or detector are necessary, and, because separations of very high resolution are possible, very pure compounds are isolated after only one chromatographic pass.

Selected applications of small-scale preparative l.c. of carbohydrates are given in Table IV. Although the amounts of carbohydrate isolated are low (several $\mu$g to 20 mg), they are often enough for subsequent qualitative methods such as $^1$H-n.m.r. spectroscopy. By computer automa-

TABLE IV

**Isolation of Carbohydrates on Analytical-Scale Columns, and Instrumentation Used**

| Class of Carbohydrate | Columns and Solvents | Specific Carbohydrates Isolated (wt. in mg) |
|---|---|---|
| Mono- and di-saccharides | HPX-87P, $H_2O$ | plant cell-wall sugars (0.5)[244] |
| | Radial Pak B, Silica gel, MeCN–$H_2O$–TEPA | sugars in standard mixture (20.0)[39] |
| Glycosides | Dextropak $C_{18}$, $H_2O$ | benzylglycosides (3000) isolated on automated system[276] methyl glycosides from synthetic reaction (8)[91] |
| Plant oligosaccharides | Dextropak $C_{18}$, $H_2O$ | linear malto-, isomalto-, xylo-, and cello-oligosaccharides (1)[60] |
| | Partisil PAC, MeCN–$H_2O$ | isomalto-oligosaccharides (2)[149] |
| Neutral oligosaccharides of animal origin | Alltech RP-5, $H_2O$; Micro PAK-AX-5, MeCN–phosphate buffer; | oligosaccharides from ovarian-cyst glycoproteins[174] |
| | Primary amine-modified silica gel, MeCN–$H_2O$ | complex or high-mannose oligosaccharides released from bronchial mucins,[172,173] ovomucoid,[179,180] ceruloplasmin and orosomucoid,[155] IgD,[168] ovalbumin,[155,167,168] and IgM[167] (samples for $^1$H-NMR-spectral analysis, mg) |
| | Dextropak C-18, $H_2O$; or MicroPAK AX-5, phosphate buffer | human-milk oligosaccharides[21,61,185] |
| Ionic oligosaccharides of animal origin | MicroPAK AX-10, phosphate buffer | sialic acid-containing oligosaccharides from ceruloplasmin,[193,197] ovalbumin,[193,197] orosomucoid,[197] IgD,[169] mucins,[196] phosphorylated oligosaccharides from β-glucosiduronase (mg levels)[193,197] |
| | primary amine-modified silica gel, MeCN–buffer | sialic acid-containing oligosaccharides from ceruloplasmin and orosomucoid,[193] IgD,[169] human milk,[192] ovomucoid,[198] mucins,[277] and sulfated oligosaccharides from glycosaminoglycans[130,131] |
| Glycopeptides | Alltech 600RP, or Supelco LC-18, phosphate buffer | protease-released glycopeptides from ovalbumin,[62] cathepsin,[204,205] and virus glycoproteins[208] |
| Microbial oligosaccharides | Partisil PAC, MeCN–$H_2O$; Spherisorb-5-ODS, MeCN–$H_2O$ | branched hexa-*O*-β-D-glucopyranosyl-D-glucitols from fungal-wall fragments (mg quantities)[165] |
| | $C_{18}$-bonded silica gel, $H_2O$ | cyclic glucans (cyclosophoroses) (mg quantities)[191] |

tion,[276] these analytical systems can be used on an unattended, 24-h basis, and gram quantities of pure carbohydrates can be obtained.

Of the many areas where these methods have been useful, the greatest impact has been in the area of complex plant and animal oligosaccharides, glycopeptides, and other glycoconjugates (see Table IV). The isolation of these pure carbohydrates, by the methods described, has allowed their spectroscopic, chemical, and enzymic analysis, in many cases for the first time (see Addendum).

**c. Preparative L.C. in Large-Scale Equipment.**—This area of preparative chromatography is in a rapid stage of development. Clearly its potential lies in the applications that require multigram (or even multi-kilogram) amounts of pure carbohydrate for research, pharmaceutical, or food applications. Formerly, large-scale, preparative l.c. required a "dedicated" instrument that used columns with "large" sample capacity (5–10 g), low resolving-capability, and requirements for "high" (200–500 mL/min) flow-rates. These inefficient systems have now been largely superseded by the use of efficient, preparative columns (2 × 30 cm), such as the Whatman Magnum 20, Dupont Zorbax Series, and Rainin Dynamax (see Section II,1,e) which allow high-resolution, "large-scale" (0.5 to 2.0 g) separations at moderate (10–25 mL/min) flow-rates. Interestingly, the efficiency of these columns is often superior to,[24,275] and the back pressure lower than, that of their analytical counterparts.

Many of the l.c. stationary-phases available may be packed into columns by the user at a fraction of the cost of a commercial column (see Section II,1,f). The most notable of these are the cation-exchange resins[8,89] and the aminopropyl-bonded silica gels,[28] which can be readily packed to provide high-resolution separations. The $C_{18}$-bonded silica gel and cation-exchange resin stationary-phases are especially useful, as large columns (2 × 25 cm) can be accommodated on analytical chromatographs, at flow rates of 1 to 5 mL/min, without any modifications of equipment.

The use of an aminopropyl-bonded-phase silica-gel column (2 × 25 cm) for the high-resolution isolation of gram quantities of malto-oligosaccharides is shown in Fig. 1. The use of a home-packed, $Ag^+$-form, cation-exchange resin, preparative column (2.2 × 30 cm) for malto-oligosaccharide isolation is shown in Fig. 2. Other specific examples of large-scale, preparative l.c. are listed in Table V. Undoubtedly, preparative-l.c. methods will be used routinely in future food-, industrial-, biological-, and biotechnological-carbohydrate applications.

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(276) A. F. Hadfield, R. N. Dreyer, and A. C. Sartorelli, *J. Chromatogr.*, 257 (1983) 1–11.
(277) G. Lamblin, A. Boersma, A. Klein, P. Roussel, H. van Halbeek, and J. F. G. Vliegenthart, *J. Biol. Chem.*, 259 (1984) 9051–9058.

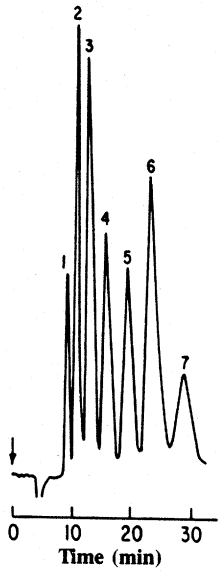


FIG. 1.—Preparative Separation of Amylose-derived Oligosaccharides on Aminopropyl Silica-gel L.c. Column (2 × 25 cm) Eluted with 11 : 9 Acetonitrile–Water at 12 mL/min. [Sample size, 500 mg; pressure, 7 MPa. Refractive index detection at 128×. Each numbered peak was collected at 86–98% purity. Numbers above peaks refer to d.p. values. See also, Ref. 8.]

TABLE V
**Isolation of Carbohydrates on Large-Scale Columns, and Instrumentation**

| Class of Carbohydrate | Columns and Solvents | Specific Carbohydrates Isolated (wts.) |
|---|---|---|
| Mono- and di-saccharides | columns (diameter 1–10 cm) packed with ion-exchange resin, $H_2O$ | neutral mono- and di-saccharides from polysaccharide hydrolyzates (100 mg)[25]; ketose disaccharides (lactulose, maltulose, cellobiulose) (200 mg)[89]; fructose (kg scale),[278,279] uronic acids[25] |
| Glycosides and simple sugar derivatives | Waters 500A, with $C_{18}$ column (5.7 × 30 cm), $H_2O$ | methyl maltosides,[5] *O*-methylated sucrose derivatives[280] (gram quantities) |
| Plant-derived oligosaccharides | Rainin aminopropyl column (2. × 25 cm), MeCN–$H_2O$ | malto-oligosaccharides, d.p. 3–7 (gram quantities)[8] |
| Neutral, animal-derived oligosaccharides | Chromatospac Prep 10 silica gel, 2-propanol–ethyl acetate–$H_2O$ | size-fractionation of high-mannose oligosaccharides, d.p. 1–9 (gram quantities)[6] |
| Acidic, animal-derived oligosaccharides | strong anion-exchange silica-gel columns (~1 × 50 cm), phosphate buffers | sialic acid-containing oligosaccharides derived from ovomucoid[198]; sulfated, uronic acid-containing oligosaccharides from heparin[216] |

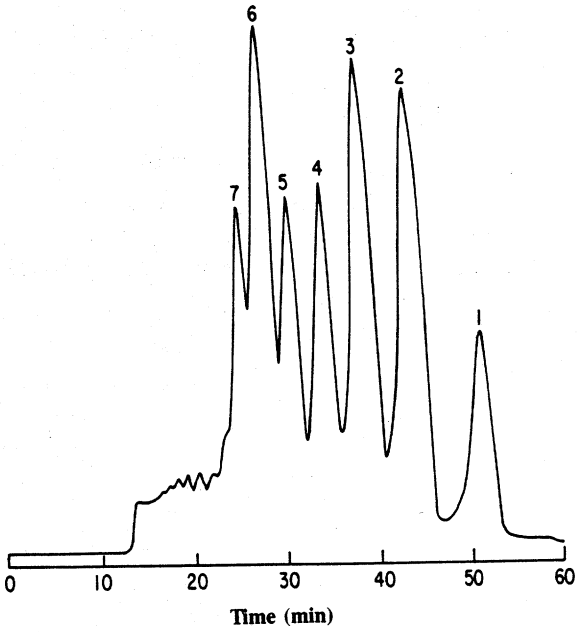


FIG. 2.—Preparative Separation of Same Sample as Used in Fig. 1, on Laboratory-packed, Preparative Column (2.2 × 30 cm) of HPX-42 ($Ag^+$) Resin, Eluted with $H_2O$ at 1.1 mL/min. [Sample size, 125 mg; pressure, 700 kPa. Refractive index detection at 128×. Each numbered oligosaccharide was collected at 75–92% purity. Numbers above peaks refer to d.p. values. See also, Ref. 8.]

## IV. Special Aspects and Problems

### 1. Detectability and Accuracy

After two decades of quantitative l.c. analyses, it has been established that, when proper precautions are taken, these methods can provide accurate and reproducible results.[229,238] Quantitative l.c. measurements are usually as accurate as, and often more precise than, those obtained by spectrophotometric,[228,241] paper-chromatographic,[83] and gas–liquid-chromatographic[227] methods. Both external and internal standardization have been used to translate peak height or areas into quantitative, solute-concentration values. Because peak heights are easy to measure, many methods use this parameter, and, when slightly overlapping peaks or unsteady baselines are encountered, it is the method of choice. With introduction of

(278) P. E. Barker and S. Thawait, *Chem. Ind. (London)*, (1983) 817–821.
(279) P. E. Barker, G. A. Irlam, and E. K. E. Abusabah, *Chromatographia*, 18 (1984) 567–574.
(280) W. Moody, G. N. Richards, N. W. H. Cheetham, and P. Sirimanne, *Carbohydr. Res.*, 114 (1983) 306–310.

the use of microprocessor-controlled, recording integrators, more peak-area analyses are being reported, especially for analysis of oligosaccharides, such as the malto-[232] and cello-oligosaccharides,[49] where each oligosaccharide gives the same peak area per unit weight (for refractive index detectors). The various detection methods used to obtain these quantitative results are described next.

**a. Refractive Index Detection.**—Refractive index (r.i.) detectors, or differential refractometers, are the most commonly used detectors for l.c. analysis of non-u.v.-absorbing, simple sugars, alditols, and oligosaccharides. These relatively inexpensive instruments are regarded as "universal detectors" because they detect almost all classes of molecules present in a mixture, and this is especially useful in preparative chromatography, where it is essential to be aware of the presence of all of the compounds in a sample. Despite their common use, these detectors have several limitations, namely, sensitivity to changes in solvent composition, temperature, and pressure. These limitations are most apparent, however, when highly sensitive detection is required. Even then, with the use of low-pulsation, solvent-delivery systems, equipped with pulse dampeners, column temperature-control compartments, and other accessories outlined in Section II,1, r.i. detection can be useful and relatively trouble-free. R.i. detectors cannot be used in conjunction with solvent gradients, and this remains one of their major shortcomings. The sensitivity of various r.i. detectors has been studied, and the limit of detection for traditional models depends upon the column and the mobile phase being used. For amine-modified silica-gel columns, mono-, di-, and oligo-saccharides may be detected at levels greater than ~5 $\mu$g.[33,39,186,242] For cation-exchange-type l.c. columns ($Ca^{2+}$ or $Pb^{2+}$ form), the detection limit is almost an order of magnitude lower,[38,39] and these columns are more useful for high-sensitivity work. In most cases, the level at which sugars can be accurately and reproducibly measured is about five times the minimal detectable levels just described. The linear range of detection differs considerably from one laboratory to another, and therefore must be determined for each instrument. Linear ranges of 5 $\mu$g to 2 mg have been reported[39,83,233,242] for various carbohydrates. Manufacturers have now introduced highly sensitive, r.i. detectors which are capable of signal-to-noise ratios that are 10 to 100 times better[281] than those of conventional r.i. detectors, allowing the detection of as little[92] as 15–25 ng of monosaccharide. With these detectors, precise analysis of sugars may be accomplished in the 200-ng to >20-$\mu$g range. A side benefit of these detectors is the dramatic lengthening of column life, due to the small amounts of

(281) J. Koops and C. Olieman, *Neth. Milk Dairy J.*, 39 (1985) 89–106.

samples (and contaminants) which are injected. At this sensitive range, however, special precautions must be taken to prevent contamination of samples, because even trace components (in samples or diluents) will produce l.c. peaks that are equivalent in size to the sample peaks.

**b. U.v. Detectors.**—U.v. detectors are useful in carbohydrate l.c. analysis, especially for those compounds having carboxyl or other u.v.-absorbing groups. Even simple alditols and sugars have been detected at low wavelengths (188–192 nm), although the sensitivity of this technique is no greater than that of r.i. detection (minimum detectable levels for these compounds are ~1–12 μg).[33,87,234,246] Owing to the low u.v.-absorbances of these carbohydrates, accurate analyses are often difficult because of interfering, chromatographic peaks from strongly u.v.-absorbing, trace contaminants in solvents and samples.[246] Carbohydrates that have higher extinction coefficients and are readily detected are as follows: (compounds, wavelength, minimal detectable level) hexuronic, aldonic acids, and lactones, 210–220 nm, 0.5–10 μg, Refs. 48 and 96; ascorbic acids, 254–268 nm, 25–50 μg, Refs. 112 and 282; sialic acids, 195–200 nm, 200 pg–6 ng, Refs. 120 and 122; oligosaccharides containing 2-acetamido-2-deoxyhexose units, 190–195 nm, 0.1–0.3 nmol[61,170]; sialylated oligosaccharides, 195 nm, 0.6 nmol[202]; and unsaturated oligosaccharides from glycosaminoglycans, 232 nm, 10 ng.[253]

**c. Miscellaneous, Direct-Detection Methods.**—Other methods for direct, l.c. detection are based on electrochemical, mass, polarographic, and flame-ionization detectors. Electrochemical detectors are most useful for such electroactive molecules as the ascorbic acids, and their application to the detection of reducing sugars is limited.[283] A modification of this technique, triple-pulsed amperometry on platinum[284] or gold[72] electrodes, has been used for the direct and sensitive (nanogram level) detection of reducing sugars, alditols, and oligosaccharides. This detector is, however, only compatible with highly basic (pH >10) mobile phases. Mass detectors, which measure light-scattering from nebulized solute, after solvent evaporation, constitute a type of universal detector that can be used with solvent gradients. Their sensitivity is about equal to that of conventional refractive index detectors when mobile phases that are low in water content are used. Salt-containing mobile-phases cannot be used. Mass detectors have been used to detect reducing and nonreducing sugars, as

(282) P. Wimalasiri and R. B. H. Wills, *J. Chromatogr.*, 256 (1983) 368–371.
(283) W. Buchberger, K. Winsauer, and C. Breitwieser, *Fresenius Z. Anal. Chem.*, 311 (1982) 517.
(284) S. Hughes and D. C. Johnson, *J. Agric. Food Chem.*, 30 (1982) 712–714.

well as oligosaccharides.[247,285–287] Polarimetric detectors[267,288] are useful in l.c., because they provide qualitative information (configuration, and enantiomeric or anomeric form) about eluted peaks. Because some sugars (arabinose, galactose, rhamnose, and mannose) occur in Nature in both the D and the L form, the determination of enantiomeric form is an important and required step in the total characterization of naturally occurring sugars or polysaccharide hydrolyzates. Polarimetric, or optical-activity, l.c. detectors have been used to determine the enantiomeric form of eluted sugars[288] and partially methylated sugar derivatives[267] (from permethylated polysaccharide hydrolyzates). The accuracy of such detectors is good, and as most l.c. solvents are optically inactive, almost any mobile phase can be used in conjunction with them. The sensitivity for these detectors is, however, quite variable, as it is related to the magnitude of the specific rotation for each sugar. For carbohydrates with $[\alpha]_D > 50°$, minimum detection limits of 100 $\mu$g have been reported.[267] For D-fructose, as little as 100 ng was detected[288] in l.c. effluents by use of a highly sensitive, optical-activity detector. Unfortunately, these detectors are not at present readily available from commercial sources. To determine absolute configuration, it is therefore necessary to use alternative chromatographic procedures wherein either indirect methods (converting the enantiomeric pair into diastereoisomers) or direct ones (chromatography on chiral, stationary phases) are used to distinguish between the D and the L form. Sensitive l.c. and g.l.c. procedures are available for these purposes and they have been reviewed.[265] Otherwise, carbohydrates eluted from l.c. columns may be collected (see Section III,3) and subsequently examined by polarimetry or circular dichroism methods[266] in order to establish the enantiomeric form. The use of flame-ionization detectors has been described.[29,81] An important feature of these detectors, which are commercially available, is their ability to monitor all types of eluted species (so-called "universal" detection). In addition, unlike refractive-index detectors, they may be used in conjunction with gradient-elution programs.

**d. Post-column Derivatization Methods.**—Often, carbohydrate samples are available in such small amounts that direct l.c.-detection by one of the previous methods is not possible. To enhance carbohydrate detectability, methods based upon post-column derivatization have been developed. In these methods, carbohydrates are separated by using normal l.c.-techniques, and only the detection system is altered. Several excel-

(285) M. Lafosse, M. Dreux, L. Morin-Allory, and J. M. Colin, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 39–41.
(286) R. Macrae and J. Dick, *J. Chromatogr.*, 210 (1981) 138–145.
(287) R. Macrae, L. C. Trugo, and J. Dick, *Chromatographia*, 15 (1982) 476–478.
(288) J. C. Kuo and E. S. Yeung, *J. Chromatogr.*, 223 (1981) 321–329.

lent reviews[29,81,289] on the subject covered advances in this area up to about 1983, and all post-column methods are now being reviewed on a biennial basis.[290,291] Table VI lists representative methods that have since

TABLE VI
**Post-Column Reagents for Enhancing the Detectability of Carbohydrates**

| Reagent | Carbohydrates | Detection Limit (method) |
|---|---|---|
| Tetrazolium Blue[34,235,292] | reducing sugars | 10 ng (P)[a] |
| Copper bis(phenanthroline)[293,294] | reducing sugars | 0.2 ng (A)[b] |
| 2-*tert*-Butyl anthraquinone[295,296] | alditols | 80 ng (F)[c] |
| Cuprammonium[297] | mono-, oligo-saccharides | 0.4 μg (P) |
| Cyanoacetamide | reducing sugars[70,79,80,298] | 0.2 μg (P) |
| | hexosamines,[80] sialic[80] and uronic[71] acids | 5.0 ng (E)[d] |
| 2,4-Pentanedione[299] | hexosamines | 0.1 nmol (F) |
| Periodate[86,300] | general carbohydrate reagent | 1.0 nmol (P) |
| Ethylenediamine[301,301a] | sugars, reducing and nonreducing | 1.0 nmol (F)<br>1.0 pmol (E) |
| *p*-Aminobenzoic hydrazide[302] | sugars, reducing and nonreducing | 1.0 ng (P) |
| Europium salts[118] | sugar phosphates | <20 μg(P) |

[a] Photometric. [b] Amperometric. [c] Fluorometric. [d] Electrochemical detection principles.

(289) R. W. Frei, H. Jansen, U. A. T. Brinkman, *Anal. Chem.* 57 (1985) 1529A–1539A.
(290) R. E. Majors, H. G. Barth, and C. H. Lochmüller, *Anal. Chem.*, 54 (1982) 347R–349R, 360R–362R.
(291) R. E. Majors, H. G. Barth and C. H. Lochmüller, *Anal. Chem.*, 56 (1984) 329R–331R, 346R–347R.
(292) R. E. A. Escott and A. F. Taylor, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 290–292.
(293) N. Watanabe and M. Inoue, *Anal. Chem.* 55 (1983) 1016–1019.
(294) N. Watanabe, *J. Chromatogr.*, 330 (1985) 333–338.
(295) M. S. Gandelman and J. W. Birks, *Anal. Chim. Acta*, 155 (1983) 159–171.
(296) M. S. Gandelman, J. W. Birks, U. A. T. Brinkman, and R. W. Frei, *J. Chromatogr.*, 282 (1983) 193–209.
(297) G. K. Grimble, H. M. Barker, and R. H. Taylor, *Anal. Biochem.*, 128 (1983) 422–428.
(298) T. D. Schlabach and J. Robinson, *J. Chromatogr.*, 282 (1983) 169–177.
(299) S. Honda, T. Konishi, S. Suzuki, K. Kakehi, and S. Ganno, *J. Chromatogr.*, 281 (1983) 340–344.
(300) P. Nordin, *Anal. Biochem.*, 131 (1983) 492–498.
(301) K. Mopper, R. Dawson, G. Liebezeit, and H.-P. Hansen, *Anal. Chem.*, 52 (1980) 2018–2022.
(301a) S. Honda, K. Enami, T. Konishi, S. Suzuki, and K. Kakehi, *J. Chromatogr.*, 361 (1986) 321–329.
(302) P. Vrátný, U. A. T. Brinkman, and R. W. Frei, *Anal. Chem.*, 57 (1985) 224–229.

been used, or developed. Most of these reagents require the use of a post-column reactor, and advantages of the various commercial and laboratory-built devices have been described.[289,302-304] A comparison of several post-column reagents has been published.[302]

**e. Pre-column Derivatization Methods.**—Another technique for enhancing the detectability of carbohydrates is to convert them into readily detectable derivatives prior to chromatography. In many cases, this should be a method of last resort, because l.c. was originally developed to analyze polar, non-derivatized carbohydrates directly. Moreover, existing methods based on g.l.c. would suffice in many applications (except for certain ones, such as oligosaccharides) where highly sensitive detection is needed. Nonetheless, there are a number of useful, pre-column derivatization procedures, and the developments in this area up to about 1983 have been reviewed[81,290,291] and methods of this type for the following carbohydrates have been described. *Simple sugars:* perbenzoates,[305] dansylhydrazones,[12,263,306] *N*-(*p*-methoxyphenyl)glycosylamines,[307] products from reductive amination with aniline,[269,270]4′-(dimethylamino)-4-aminoazobenzene,[264] or 2-aminopyridine[308]; *alditols:* perbenzoates,[309] phenylisocyanates[310]; *glycosides:* perbenzoates,[261] per-*p*-bromobenzoates and pernaphthoates[266]; *sialic acids:* (*p*-nitrophenyl)hydrazones[311]; *hexosamines:* products from reductive amination with 2-aminopyridine[272,308] or 4′-(dimethylamino)-4-aminoazobenzene[264]; *uronic acids:* products from reductive amination with 4′-(dimethylamino)-4-aminoazobenzene[264]; *glycosaminoglycan-derived oligosaccharides:* product from reductive amination with aniline[256,312]; and *simple and complex oligosaccharides:* perbenzoates,[15,313] dansylhydrazones,[12,306] products from reductive ami-

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nation with 2-aminopyridine,[269,270] 4′-(dimethylamino)-4-aminoazobenzene,[264] 2-aminopyridine, and 7-amino-1-naphthol.[314]

## 2. Combined L.C. Techniques (L.C.–M.s. and L.C.–N.m.r. Spectroscopy)

The separative power of l.c. can be coupled to various types of instruments in order to obtain additional qualitative information about eluted compounds. Two such instruments that may be coupled to l.c. include n.m.r. and mass spectrometers. Although there has been one report on the use of l.c.–n.m.r. spectroscopy[315] in carbohydrate research, it is the l.c.–mass spectrometry (m.s.) system that has received the bulk of the attention. Mass spectrometers are capable of generating a great deal of information about molecular weights, empirical formulas, isotope ratios, and molecular structure, but the actual on-line coupling of m.s. to l.c. has been a challenge, and the full potential of this method has yet to be realized. To begin with, l.c. requires high-pressure, liquid-phase operation, whereas m.s. requires gas-phase and vacuum operation. Despite these diametrically opposed principles, several interfaces have been developed to allow on-line m.s. detection of l.c. effluents, and these have been reviewed.[316–319] The direct, liquid introduction of l.c. effluents into m.s. ion-sources (one type of interface) creates a huge burden on the vacuum system, and hence, only 1–3% of the total flow from a conventional l.c. column may be directly injected. Although the sensitivity of such split-stream interfaces is low, they have been used to analyze eluted sialic acids,[121] and various peralkylated oligosaccharides.[166,273] When microbore columns are used, the flow rates are low (<100 μl/min), and the entire column-effluent may be injected without stream splitting, allowing more-sensitive detection and analysis of simple sugars.[320] Thermospray[57e,317,321] and heated nebulizer[318] interfaces allow the direct coupling of conventional l.c. columns with moderate flow-rates (of salt-free, mobile phases) to either normal or atmospheric pressure, chemical-ion-

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ization mass spectrometers.[318] These methods are useful for analyzing such small, polar molecules as peptides and sugars.

An alternative to direct liquid introduction is the moving belt, or moving-wire, transport interface.[319] Because all l.c. solvents are evaporated before the sample is transported into the ion source, fewer restrictions are placed on solvent type, flow rates, or buffer composition. This system has been used for analysis of mixtures of pentoses, hexoses, and disaccharides.[319]

Most of the direct and indirect (transport) interfaces described here use chemical ionization (c.i.) ion-sources, which are not well suited to such polar, non-volatile compounds as tri- and higher oligosaccharides. The thermospray interface, which can operate on an ion-evaporative mode,[317] is capable of producing intact molecular ions from such nonvolatile, polar molecules and should be useful in oligosaccharide analysis. Molecules of this type, however, can also be easily analyzed by fast-atom-bombardment ionization, and use of this technique,[321a] coupled to direct liquid introduction[322] and moving-belt[323] interfaces, has been reported. The latter system has been applied to complex oligosaccharide analysis.[324]

### 3. Separation of Carbohydrate Anomers

Although reducing sugars exist in solution as a mixture of two or more tautomeric forms, they often give only one peak when analyzed by l.c., and this is fortunate for analytical purposes. There are, however, instances where the separation of sugar anomers is encountered. Partial or complete separation of anomers of reducing sugars and oligosaccharides has been achieved by ligand-exchange chromatography on cation-exchange resins (metal form),[53,267a] by normal phase-partition chromatography on either polystyrene-based anion-exchangers (sulfate form)[325] or aminopropyl-bonded silica gel (in the sulfate[135] or acetate[326] form), by combined partition–ligand exchange chromatography on $Na^+$- and $Ca^{2+}$-form cation-exchange resins[78], and by reversed-phase chromatography on alkyl-bonded silica.[60,64,69] In all these cases, anomers of individual reducing sugars are separated because their mutarotation rate is low, relative to the speed of the chromatography. Pyranose anomers of some sugars may

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be separated at room temperature on one system,[53] but, by using a lower temperature (0–4°), nearly all of the $\alpha$- and $\beta$-pyranose anomers of the common aldohexoses and aldopentoses can be separated.[78] Furanose anomers interconvert at higher rates, and temperatures of $-25$ to $-45°$ and special solvents were necessary in order to separate the $\alpha$- and $\beta$-furanose anomers of L-fucose and D-galactose.[326,327] Because of the rapidity of these assays, and their accuracy, as verified by n.m.r. spectroscopy[78] and polarimetry,[135,326] they have been used to determine the mutarotation rates of the common sugars.[78,326]

When resolution of anomers is not desired, it can usually be prevented by the following procedures. Cation-exchange columns (metal forms) should always be run at temperatures $\geq 85°$. Aminopropyl-bonded silica-gel columns should not be used in the acetate or sulfate form. On alkyl-modified silica, the prevention of anomer separation is not easy, and increasing the temperature, or adding catalysts to increase the mutarotation rate, causes loss of retention.[69] When sugars and oligosaccharides must be analyzed on such columns, they should be reduced by sodium borohydride prior to chromatography, in order to obtain sharp and symmetrical, chromatographic peaks.

## 4. Future Trends

New advances in the l.c. of carbohydrates are likely to come from three general areas. The first is in the development of more-durable and stable, stationary phases. At present, a major limitation on the use of commercial columns, especially those of the aminopropyl-bonded silica-gel variety, is their short life-time and ease of fouling. More-durable, resin-based columns that operate with the same solvent system and selectivity as aminopropyl silica-gel columns are currently available,[80] and will see further use and development. The development of improved phases for supercritical, fluid-type l.c. will allow this method to be of use for analysis of various carbohydrates.[327a]

A second field of rapid development in the l.c. of carbohydrates is in practical, preparative chromatography. Early preparative systems used large, expensive columns with low resolving power, and hence, were not extensively applied in carbohydrate research. New research[8] is showing that various carbohydrates can be separated on the gram scale, using normal l.c. equipment and large columns home-packed with relatively

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(327a) T. L. Chester and D. P. Innis, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 209–212.

inexpensive resins and bonded silica gels. Rapid advances are to be expected in this area (see Addendum).

A third area of development in carbohydrate l.c. analyses is in the combined techniques (see Section IV,3) and other methods that provide qualitative, as well as quantitative, information about sample constituents, such as high-performance liquid affinity chromatography. The use of specific lectin- and monoclonal antibody-based, stationary phases for analytical and preparative applications is now being considered. The basic concepts of these techniques have been reviewed[328,329] and their applications to carbohydrates[1,330] have been discussed.

## ACKNOWLEDGMENTS

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# ADDENDUM TO ARTICLE 3: REFERENCES PUBLISHED AFTER 1986 (ADDED AT PROOF STAGE)

BY KEVIN B. HICKS

| GENERAL AREA | SPECIFIC TOPIC AND REFERENCE |
|---|---|
| **Review articles** | Simple and complex carbohydrates[331] |
| **Instrumentation and stationary phases** | Laser-based refractive index detectors,[332] "Diol" silica gel phases,[333] Monoclonal antibody-based stationary phases[334] |
| **Separations** | |
| Neutral mono-and di-saccharides | Kestoses and nystose,[335] Fermentation-derived sugar acids,[336] Ascorbic acid-2-phosphates,[337] Sugar phosphates,[338] Inositol phosphates[339,340] |
| Ionic mono- and di-saccharides | |
| Simple, neutral oligosaccharides | Gluco-oligosaccharides,[333,341,342] |
| Simple, ionic oligosaccharides | Polysialic acids,[343] Oligogalacturonic acids[343a] |

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| Complex, neutral oligosaccharides | Cyclic oligosaccharides,[342,344–346] N-linked oligosaccharides,[347,348] |
| Complex, ionic oligosaccharides | Heparin fragments[349] |
| **Applications** | |
| Analysis of food carbohydrates | Nanogram detection of sugars,[350] Lactulose,[351] Pectins[343a] |
| Analysis of carbohydrates in biomass conversion processes | Sugars, oligosaccharides, and degradation products,[341,352,353] Pectins[343a] |
| Compositional analysis of carbohydrate polymers | Proteoglycan-derived carbohydrates,[354] Lipopolysaccharides,[355] Glycoproteins,[356] Food Polysaccharides[343a,353] |
| Structural and sequence analysis of carbohydrates | N-Linked oligosaccharides,[347,348] Degree of polymerization of neutral oligosaccharides,[357] Degree of methylation and acetylation of pectin,[358] "High mannose" oligosaccharides[359] |

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| **Preparative l.c.** | $^{14}$C-Labeled oligosaccharides, sugars, and sugar degradation products,[341] Trehalulose,[360] General methods for mono- and disaccharides and sugar acids,[361] Kestoses and nystose,[335] Chitin oligosaccharides[362] |
| **Special aspects** | |
| Detectability and accuracy<br>Post-column detection methods | Laser-based refractive index detector,[332] Cuprammonium reagent,[333,363] 4-Aminobenzoic acid reagent,[350] Indirect detection methods for cyclodextrins,[345] and sugar phosphates[338] |
| Pre-column derivatives | Reversible derivatization using 2-amino-pyridine[364] |
| Combined l.c. techniques | Direct coupling of l.c. to f.a.b.m.s. for analysis of oligosaccharides[365–367] |
| Future trends | Capillary l.c. of sugars,[332] Affinity separations of oligosaccharides[334] |

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